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Improvement of Methods for Differentiating Species of *Brucella*: *B. abortus* and *B. melitensis* Using Real-Time PCR

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Abstract

In this article the comparison of different methods for typing *Brucella* spp. A technique for different species *B. abortus* and *B. melitensis* based on real-time polymerase chain reaction (PCR). Defined a method to more quickly typed bacteria of the genus *Brucella*. PCR in a short time PCR-analysis has spread around the world, quickly leaving the laboratories of scientific institutions in the sphere of practical clinical use. Diagnosis of infectious diseases, including those caused by agents that are difficult to cultivate, genotyping microorganisms, evaluation of their virulence, the definition of stability microorganism to antibiotics, gene diagnostics, prenatal diagnosis, biological control of blood, and more other the successfully PCR, is used. The main stage of recovery from infectious animal diseases, including brucellosis, is timely and rapid diagnosis, which occupy an important place laboratory tests for the detection of pathogens in pathological material the main goal of the research, was to determine the methods for rapid detection of *Brucella* spp. To solve the problem of goal were: pick primers specific to strictly *Brucella* spp., and optimize real-time PCR conditions for a specific region of the genome *Brucella* spp. The objects of the study were blood from three cattle and three small ruminants – sheep (n = 6), react positively to brucellosis on the results of serological tests RBP and AR. Work was carried out on the basis of the Research Institute (Laboratory of Biotechnology Engineering profile) WKATU named after Zhangir Khan. Real-time PCR was carried out on the device company iQ5 BioRad. DNA extraction was performed by cetyltrimethylammonium bromide (CTAB). The quality of DNA extraction was determined by the method of agarose gel electrophoresis.

Keywords

PCR; *Brucella* spp.; *Brucella abortus*; *Brucella melitensis*; *Brucella ovis* differentiation

Introduction

Traditional differentiation methods of various *Brucella* species are very laborious and time-consuming, and are not always reliable. For example, certain strains of different species of *Brucella* can be absolutely identical biochemical properties. So, need to search for significant differences. Responsible for such differences carries the DNA of microorganisms directly. In the last 20 yrs, the world of microbiology has seen a revolution. This happened largely due to the rapid development and implementation in practice molecular biology techniques [1,2].

Research is needed due to the pressing problems of modern veterinary medicine, when it becomes apparent that brucellosis being anthropozoonosis represents a danger to livestock and humans. For this reason, research on methods to differentiate between the different *Brucella* species based on real-time PCR method should be an integral part of a new, practice-oriented diagnosis of brucellosis. If we take into account the fact that the vast majority of cases of brucellosis is not subject to species differences, it becomes obvious that the only way an adequate analysis are methods of modern molecular biology, the use of which is addressed in this study [3-6].

In modern veterinary science and practice of detection and differentiation of pathogens, brucellosis conducted using serological tests, consuming and takes a lot of time studying the culture-biochemical traits on nutrient media, where there is a need for differentiation of carbon dioxide in the formation of hydrogen sulfide, etc. To date, the known following types of *Brucella*: *Brucella melitensis* (Maltese), *Brucella abortus bovis* (bovine), *Brucella abortus suis* (pork), and *Brucella canis* (dog) (Table 1).

In recent years, molecular biology has been increasingly used PCR, DNA amplification by PCR, it became one of the main methods in experimental studies to help solve a variety of tasks in research. Bulat implemented genetic type of Yersinia pseudotuberculosis strains by PCR using oligonucleotide primers 5-22 nucleotides in length, which were synthesized based on the nucleotide sequences of the hypervariable regions of the DNA of M13 phage. PCR was originally developed for the amplification and analysis of specific genetic loci, and based on the prior knowledge of the nucleotide sequences of these loci in the modified embodiment, using universal primers was suitable for a detailed analysis of the genomes of all organisms. New method is a necessary prerequisite for the development of any branch of science. They allow to obtain previously unavailable information, which in turn leads to a deeper understanding of the essence of the observed phenomena and stimulate further research, generating new discoveries.

PCR is one of the widely used techniques of molecular biology, which is used for a significant increase in the concentration of certain nucleic acid fragments in the sample. The method is a certain copy of a DNA region with the use of various enzymes *in vitro*. PCR consists of three consecutive steps (denaturation, amplification, and elongation).

The principle of the PCR was developed by Cary Myullisom (firm "Cetus", USA) in 1983, opening the PCR has become one of the most significant developments in the field of molecular biology over the

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Types	Disease
Brucella melitensis (biovars 1-3)	Brucellosis goats, sheep, human
Brucella abortus (biovars 1-6,9)	Brucellosis of cattle, human
Brucella suis (biovars 1-5)	Porcine brucellosis, human
Brucella canis	Brucellosis dogs
Brucella ovis	Brucellosis sheep (epididymitis in rams)
Brucella neotomae	Brucellosis rats, guinea pigs, mice

Table 1: Types of *Brucella*

past 20 yrs. For development of PCR, Myullisom in 1993 was awarded the Nobel Prize in Chemistry. Appearance of PCR was due to certain advances in molecular genetics, especially decoding the genomes of a number of nucleotide sequences of microorganisms cannot say that PCR was made possible by the discovery of a unique enzyme Taq–DNA–polymerase contained the bacteria that live in geysers. Feature of this polymerase is its exceptional heat resistance (can withstand heat up to boiling temperature without loss of activity), and high operating temperatures (optimum operation – 72°C).

Elegance, simplicity, performance, unsurpassed sensitivity, and specificity of the new method yielded enormous popularity. In a short time, PCR-analysis has spread around the world, quickly leaving the laboratories of scientific institutions in the sphere of practical clinical use. Diagnosis of infectious diseases, including those caused by agents that are difficult to cultivate, genotyping microorganisms, evaluation of their virulence, the definition of stability of microorganism to antibiotics, gene diagnostics, genetic fingerprinting, prenatal diagnosis, and biological control of blood – this is not a complete list of areas where he successfully PCR is used.

Identification of an organism in the study usually occurs on resistance genes by the promoter as well as the genes. Distinguished: conserved genes, such sequences that vary slowly in continuous evolution. Conserved genes encode the genetic apparatus of the cell and analyzing conserved genes in organisms, we can identify the body classes. With the help of gene research, the average conservatism body to the family can be determined. Allocate the same genes low conservatism and highly variable genes on them the body can be determined to species.

The aim of our research was to improve the method of differentiation of various *Brucella* species by the method of molecular biology real-time PCR [7,8].

Materials and Methods

Work was carried out on the basis of the Research Institute (Laboratory of Biotechnology Engineering profile) WKATU named after Zhangir Khan. PCR was carried out on the device company iQ5 BioRad.

As objects of study were bled from three cattle and three small ruminants, sheep ($n = 6$) react positively to brucellosis on the results of serological tests RBP and AR.

To differentiate types of *Brucella B. abortus* and *B. melitensis,* bacteriostatic method is used – differentiation in resistance to aniline dyes. For differentiation, these cells were used, consisting of meat water, NaCl, peptone agar, and dyes, as basic fuchsin and thionin 1:50,000 1:25,000.

To carry out molecular biological studies of biological samples, typical experimental setup was used. Scheme of a typical experiment involves the separation of total DNA from the sample, PCR amplification of specific regions of the genome.

In the process of isolating DNA from biological samples, standard laboratory equipment was used for work on molecular microbiology, such as automatic dispensers, centrifuges, electrophoresis equipment, visualization and documentation of gels, refrigerators, freezers, vortices, etc.

DNA extraction was performed by CTAB. The quality of DNA extraction was determined using the method of electrophoresis in agarose gel. Whole blood was taken from the animals in the test tubes with 3% EDTA based 10:1. DNA was isolated from blood using the extraction buffer consisting of 2 g of CTAB (detergent destroys cell membranes, forms complexes with proteins and polysaccharides acid), 28 ml of 5M NaCl, 4 ml 0.5M EDTA (pH 8.0), distilled water, adjusted to 100 ml. To 100 μl of blood, 300 μl extracting buffer was added and incubated at 60°C for 1 h, the tube contents are periodically stirring. After incubation, an equal volume of chloroform, and left for 1 h at room temperature. Tube contents were stirred continuously. Then centrifuged 5 min at 5,000 rpm. The upper phase was transferred to a clean tube and add 2/3 volume of isopropyl alcohol were mixed. The tubes with the contents were kept at room temperature for 2 h to precipitate the DNA. Then centrifuged for 10 min at 12,000 rpm. The supernatant was decanted and the precipitate was washed with 70% – ethanol, centrifuged under the same conditions. Then, the liquid was poured over the sediment, the sediment was dried and dissolved in water-free DNA and RNA.

The main essence of the methods is that we must first remove the cell membranes, denature proteins associated with DNA, remove impurities, and precipitate directly allocate already purified DNA.

Quality was determined using DNA isolated by agarose gel electrophoresis. Agarose gel electrophoresis is used in molecular biology for separation of molecules (proteins and nucleic acids, and fragments thereof) by an electric field according to their masses and spatial structure [9,10].

Finding of primers was performed according to literature data, and based on computer analysis using the software package Vector NTI. Species identify that ability primers were determined using the tool "Blast" (http://blast.st-va.ncbi.nlm.nih.gov/).

Results and Discussion

During the research, it was revealed that the blood be taken from the cow species bacterium *Brucella abortus*, and sheep's blood type bacterium *Brucella melitensis* (Table 2).

The studies determined that on nutrient media supplemented with growth characteristic fuchsine, only *B. abortus* and *B. melitensis* are significant characteristic growth on nutrient media with the addition of magenta and not significant on media supplemented with Lauth's violet, and seeding with the blood of cattle on growth media with Lauth's violet not allowed. Reading of the results was carried out after six days.

In the course of research on the selection of strictly specific primers for species, *Brucella* species *B. melitensis*, *B. abortus*, *B. ovis* were identified and were picked up by three pairs of primers:

1. Ba si F (5′-TGCAGCTCACGGATAATTTG-3′), Ba si R (5′-GTTGTGGAAGGTGAGTGTGCCCGC-3′).
2. Bm/o si F (5′-CAATATCGCATCGGCAGTTTC-3′), Bm/o si R (5′-TGATACCAGCTTTTGGCC-3′).
3. Bo si F (5′-TGACGAGGCGCTTGATGATA-3′), Bo si R F (5′-TGATTTCAAGCCATTCCAGG-3′).

Dye	Crops from animals react positively to brucellosis in the formulation of AR and RBP	
	Cattle blood	Sheep blood
Fuchsine 1:50,000	+++	++
Lauth's violet 1:25,000	0	+

Table 2: Results of bacteriological differentiation *Brucella*

Name of species	Name of the strain according to the nomenclature*	Identification number*	PCR 1 (Ba si)	PCR 2 (Bm/o si)	PCR 3 (Bo si)
B. melitensis	bv. 2 str. 63/9	CP007788.1	–	+	–
	bv. 1 str. 16M	CP007762.1	–	+	–
	bv. 3 str. Ether	CP007761.1	–	+	–
	str. NI	CP002932.1	–	+	–
	str. M5-90	CP001852.1	–	+	–
	str. M28	CP001489.1	–	+	–
B. abortus	strain ZW053	CP009099.1	+	–	–
	strain 3196	CP007708.1	+	–	–
	bv. 2 str. 86/8/59	CP007764.1	+	–	–
	strain 63 75	CP007662.1	+	–	–
	strain BFY	CP007737.1	+	–	–
	bv. 6 str. 870	CP007710.1	+	–	–
	bv. 9 str. C68	CP007706.1	+	–	–
	strain NCTC 10505	CP007701.1	+	–	–
	strain BER	CP007683.1	+	–	–
	strain BDW	CP007680.1	+	–	–
	A13334	CP003177.1	+	–	–
	S19	CP000888.1	+	–	–
	biovar 1 str. 9-941	AE017224.1	+	–	–
B. ovis	ATCC 25840	CP000709.1	–	+	+

*According to the National Center for Biotechnology Information USA.

Table 3: Species identifies the ability of primers for the identification and differentiation of *Brucella* species: *B. melitensis*, *B. abortus*, and *B. ovis* by real-time PCR

In the analysis on the species identifies the ability matched primers was determined strict specificity pairs Ba si for *B. abortus* and Bo si for *B. ovis* (Table 3).

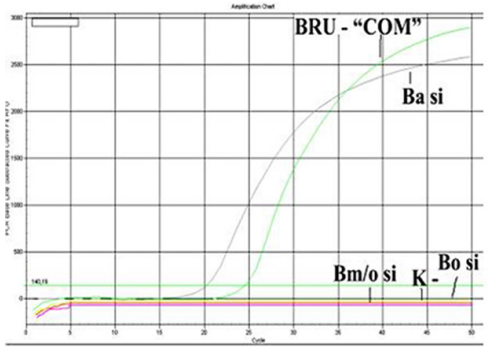
As can be seen from the table, primer pair Bm/o si defines marker portion of *Brucella* species: *B. melitensis* and *B. ovis*; in this case, the determining factor will be conducting PCR with primers Bo si with a positive reaction can be seen that in a sample to be *Brucella* species *B. ovis*, at a negative *B. melitensis*, as primers Bo si have a negative affinity for any strain of *Brucella* species *B. melitensis*.

The optimization of PCR conditions raised a number of experiments with selected primers and programing with a temperature gradient which consists of 50 cycles comprising Stage 1 "denature" at 95°C for 15 s, Stage 2 "amplification" at a temperature of 52 to 58°C for 20 s and three phase "elongation" or "synthesis" at 72°C for 30 s. Resulting PCR with temperature gradients in "real time" is defined PCR program to identify *B. melitensis*, *B. abortus*, *B. ovis* and consisting of 50 cycles comprising Stage 1 "denature" at 95°C for 15 s, stage 2 "amplification" at a temperature of 56°C for 20 s and three stages "elongation" at 72°C for 30 s.

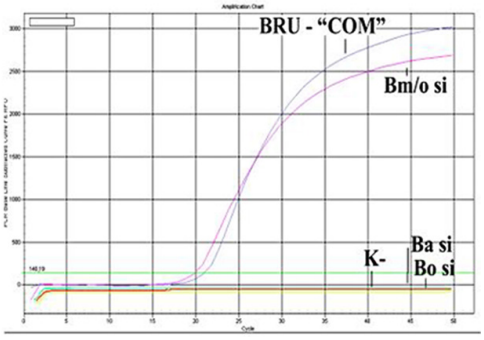
To determine the species-specific primers ability, we set a number of real-time PCR:

1. DNA isolated from the vaccine strain *B. abortus* 82 and primers Ba si, Bm/o si, Bo si and commercial set "BRU-KOM" (Figure 1A).
2. DNA extracted from the vaccine strain of *B. melitensis* Rev-1 and primers Ba si, Bm/o si, Bo si and commercial set "BRU-KOM" (Figure 1B).
3. DNA isolated from a museum strain of *B. ovis* and primers Ba si, Bm/o si, Bo si and commercial set "BRU-KOM" (Figure 1C).

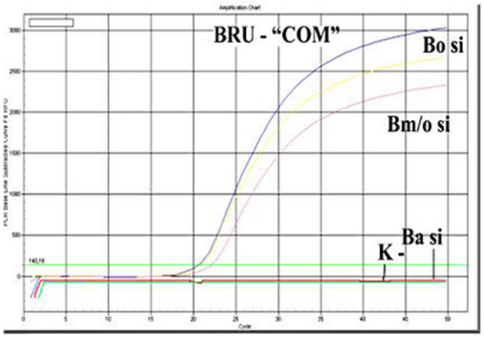
Thus, in the course of real-time PCR primers revealed a strict specificity Ba si bacteria species *B. abortus* (Figure 1A), the primers Bo si bacteria species *B. ovis* (Figure 1C) and Bm/o si bacteria species *B. melitensis* and *B. ovis* (Figure 1B). Commercial kit "BRU-KOM" in



(A)



(B)



(C)

Figure 1: The results of a species identification ability primers for real-time PCR

A – PCR with DNA vaccine strain *B. abortus* 82; B – PCR with DNA vaccine strain *B. melitensis* Rev-1; C – PCR with DNA museum strain *B. ovis*

this case was rodospetsifichen that meets the specifications stated in the instruction manual.

When conducting research with field strains, selected primers behaved similarly and fully took on the identity of differentiation with the traditional method.

Conclusion

Methodology differentiation of bacteria by real-time PCR demonstrated its efficiency and accuracy compared to the more laborious method of cultivation of bacteria and biochemical typing. Real-time PCR can be used successfully veterinary specialists for diagnosis and differentiation of various types of *Brucella*.

In the course of the research, species were chosen to identify primers for bacteria of the genus *Brucella*. It was found that the method based on the method of real-time PCR allows us to differentiate *B. melitensis*, *B. abortus*, *B. ovis* from other species of the genus *Brucella* without using cultural methods in a shorter time, which is important for practical veterinary science and medicine. This approach can be applied to epizootic brucellosis monitoring to detect pockets of a species of *Brucella* across the district, region and the state, which will allow a better understanding of the process of epizootic spread of brucellosis.

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