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Research paper



Investigating Nano particles in Bioinformatic Analysis of the Brucella Genome for Indication and Differentation by qPCR

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Abstract

Based on the identified genes, the design of specific primers and probes for qPCR was made, which makes it possible to differentiate *B. melitensis* and *B. abortus* in a single reaction. For the differentiation of Brucella strains of the same species, the PCR method of multilocus VNTR analysis, optimized for uniform temperature conditions, is described. In addition, MLVA profiles for *B. abortus* and *B. melitensis* genomes are presented. All methods of indication and differentiation of Brucella species and strains are described in detail, which allows them to be used in the everyday work of any PCR laboratory dealing with brucellosis. Control of amplification in the indication of bacteria of the genus Brucella and determination of their belonging to *B. abortus* and *B. melitensis* species is recommended to be carried out using the plasmid DNA designed in this work.

Keywords: *Brucella; genome; indication;nano particle; identification; qPCR; MLVA.*

1. Introduction

There is a wide variety of species of the genus Brucella found in nature [1]: *B. melitensis; B. abortus; V. suis; B. neotomae; B. ovis; B. canis; B. ceti; B. pinnipedialis; V. microti.* To date, there are methods for laboratory diagnosis of brucellosis pathogens by molecular genetic methods [2]. New approaches are being introduced in the indication and identification of antigenic, vaccine and field Brucella spp. using multilocus sequence typing [3], Luminex bead-based suspension array [4], Multiple-locus variable number tandem repeat analysis (MLVA) [5]. The methodology of PCR for the detection of brucella in organs and tissues in postmortem studies is being improved [6]. In addition, there are differentiation methods for different strains of the same species, based on the Variable number tandem repeat analysis (VNTR) in the genome of brucella and other organisms [7]. Such studies supplement various databases on the genomes of infectious disease pathogens, one of which is BrucellaBase, which contains information on the genomes of more than 500 strains of Brucella [8]. In this paper we showed the available tools for gene diagnosis and differentiation of brucellosis pathogens.

2. Materials and Methods

For the genetic indication of bacteria of the genus Brucella and optimization of the methodology for their differentiation, a bioinformation analysis of the brucella genome of various species and strains was carried out, the general principles of bioinformatic analysis are similar to the development of primers for other biopathogens [9, 10]. Nucleotide sequences for genome analysis were obtained from the National Center for Biotechnology Information (NCBI), in addition, "BLAST" and "VectorNTI 9.1.0" (Invitrogen Corporation) software utilities were used.

The design of oligonucleotide primers for quantitative real-time PCR (qPCR) was performed with the expectation of the possibility of multiplex amplification of various DNA markers in a single reaction. For the control of qPCR, a positive DNA sequence was developed comprising all of the desired marker nucleotide sequences. To amplify the chromosomal DNA, nucleic acids isolated from bacterial cultures of various brucella species (*B. suis*, *B. abortus*, *B. melitensis*) were used in the work. Isolation of nucleic acids was carried out by the method of magnetic sorption with a set of reagents "Magno-Sorb" variant 100-200, according to the instructions of the manufacturer – "Amplisens" (Russia).

For qPCR amplification, the following composition of the reaction mixture was used, per one sample: 1.5 µl of a 25 mM $MgCl_2$ solution; 2 µl of a 2.5 mM dNTP solution; 1.5 µl of 10x PCR buffer; 0.5 µl of 10 pM probe solution for PCR; 10 pM solution of forward and reverse primers of 0.5 µl; 0.5 µl Taq polymerase; 5 µl DNA and 3.5 deionized water. All the reagents were produced by Syntol (Russia). The final volume of the reaction mixture was 15 µl. The probe for PCR, forward and reverse primers were applied individually (specifically) for each type of PCR (Table 1) and in the multiplex variant (indication of generic and specific DNA markers in one reaction mixture). The reaction mixture for each PCR in MLVA was identical, except for the probe for PCR, not used in this reaction, and 10x PCR buffer with EvaGreen stain (instead of the 10x PCR buffer described above) was used to monitor PCR amplification.

PCR was performed on a C1000 amplifier with an optical unit CFX96 (BioRad). The amplification program was as follows: (i) DNA denaturation at 95°C for 2 minutes; (II) 5 cycles consisting

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of: 10 seconds at 95°C, 30 seconds at 60°C, 10 seconds at 72°C; (III) 40 cycles consisting of: 10 seconds at 95°C, 30 seconds at 60°C, 10 seconds at 72°C. Detection of the result of PCR (fluorescence) occurs each 40th cycle of the third stage of the PCR, at 60°C via the FAM, Rox and R6G channels.
Electrophoresis was performed in a chamber for vertical electrophoresis (glass size 100×100 mm) manufactured by Helicon, with supply of 120 V into the chamber for 180-300 minutes. The buffer used for electrophoresis was from the "EF" kit (Central Research Institute of Epidemiology, Russia).

3. Results and Discussion

3.1 Analysis of Brucella Genes

The study on selecting the most informative genes for gene indication involved analysis of genomes of various Brucella strains of species *B. abortus*, *B. melitensis*. The alignment of the nucleotide sequence of different strains and isolates of each gene and the search for the genus-specific and species-specific sequences revealed optimal nucleotide sequences that can be successfully used for gene identification of bacteria of the genus Brucella and differentiation of Brucella species of interest to us (*B. abortus* and *B. melitensis*). Thus, for the indication of the genus Brucella, the gene BSCP31 (the sequence cipher in the gene bank – KX529834) was chosen, with a molecular mass of 1034 bp localized in the first chromosome. The locus BAW_20982 with a molecular mass of 1220 bp was chosen as the optimal display for *B. abortus*, the locus is located on the second chromosome at 1003355 to 1004575 bp (strain Wisconsin). For the indication of *B. melitensis*, the locus C0R52_12390 with a molecular mass of 482 bp, localized in the second chromosome at 906042 to 906524 bp (strain B3), showed to be the most specific, and was used for the further design of the primers and a probe.

3.2 Design of Primers and Probes

Based on the nucleotide sequence of the above marker loci, the design of primers and probes for PCR was performed (Table 1); the main criteria were: to achieve the most identical annealing temperature of the primers; minimum content of dimers and secondary structures in primers and probes; a minimum of guanine and cytosine at the 3' end of the primers; the absence of guanine at the 5' end of the probe.

Table 1:. Nucleotide sequence of primers and probes for PCR indication of brucella

Detectable pathogen	Primer / probe encoding	5 '-> 3` nucleotide sequence
genus Brucella	Bru F	tcgaatggctcggttgcca
	Bru R	cgggtaaagcgtcgccagaa
	Bru P	(Fam)cgatcaagtcgggcgctctgga(BHQ1)
B. abortus	Bru abortus F	gcaatcgtcgtattgccactataatcat
	Bru abortus R	gacggcgcagttctcgaacaa
	Bru abortus P	(Rox)ccgaaaggatcagcgtgccagaa(BHQ2)
B. melitensis	Bru meli F	tgcttggcacctcggaaacac
	Bru meli R	cattcccgaaagccgatagagtttga
	Bru meli P	(R6G)tgaagcgctgcagacaaattttgacttcc(BHQ2)

For the convenience of interpreting the results of PCR with the above primers and probes, the latter were labeled with various fluorescent stains, which are also indicated in Table 1, in parentheses. The characteristics of the primers and probes resulting from the design are indicated in Table 2.

Table 2:. Characteristics of the PCR-intended primers and probes

Detectable pathogen	Primer / probe encoding	Melting point	Number / length of dimers	Number / length of secondary structures
genus Brucella	Bru F	59.6°C	5 / 3 and 4	2 / 3 and 4
	Bru R	59.7°C	2 / 3	0
	Bru P	65.1°C	4 / 3 and 4	1 / 3
B. abortus	Bru abortus F	59.5°C	7 / 3, 4 and 6	3 / 3 and 6
	Bru abortus R	59.8°C	4 / 4	0
	Bru abortus P	64.0°C	1 / 4	0
B. melitensis	Bru meli F	59.6°C	2 / 3	1 / 3
	Bru meli R	60.2°C	4 / 3	1 / 3
	Bru meli P	66.6°C	7 / 4 and 6	1 / 4

As can be seen from Table 2, the developed primers are characterized by the same melting point (59.73±0.23°C), which allows for amplification under unified reaction conditions (one amplification program), from one to seven dimers and secondary structures in primers and probes, without the formation of critical polyneomas for PCR. This in aggregate will allow the use of these primers and probes for the indication of the brucella DNA. Complementarity of the above primers and probes (Table 1) to the nucleotide sequences of the desired bacteria is shown in Figure 1.

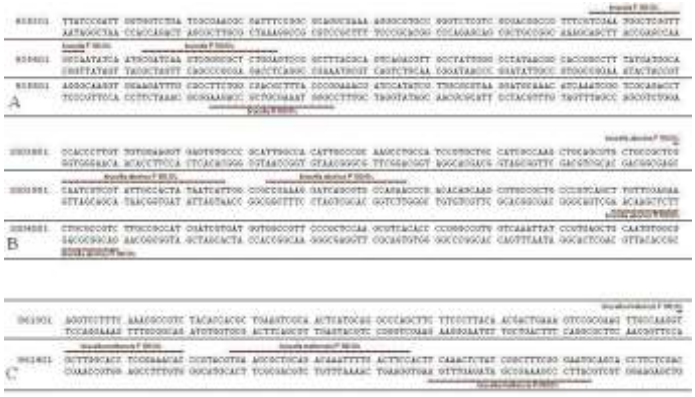


Fig. 1:. Complementarity of primers and probes for the indication of bacteria of the genus Brucella and species *B. abortus* and *B. melitensis*: A - nucleotide sequence of the gene BSCP31 *B. canis* strain YH-C16; B - nucleotide sequence of the locus BAW_20982 *B. abortus* strain Wisconsin; C - nucleotide sequence of the locus C0R52_12390 *B. melitensis* strain B3.

Primers and a probe for the indication of bacteria of the genus Brucella (in Figure 1 denoted as brucella F, R, P) amplify a gene characteristic of all genus species, including *B. abortus* and *B. melitensis*. Primers and a probe for the indication of bacteria belonging to species *B. abortus* (in Figure 1 denoted as brucella abortus F, R, P) are complementary only for different strains and isolates of this species. Primers and a probe for indicating bacteria belonging to species *B. melitensis* (in Figure 1 denoted as brucella melitensis F, R, P) are also characterized by specific amplification of bacteria of this species only.

3.3 Design of Primers for Multilocus VNTR Analysis

Marker loci for MLVA of bacteria of the genus Brucella were determined based on the analysis of VNTR loci of brucella presented on the website: http://mlva.u-psud.fr/mlvav4/genotyping/query.php. The nucleotide sequence of VNTR loci is determined from the data published by S.O. Sadikalieva and co-authors [7]. For the convenience of the reaction, the primers were modified, the nucleotide sequences are shown in Table 3. This modification was performed to optimize the molecular weights of the amplification products obtained (for convenient analysis of the amplicon after Polyacrylamide Gel

Electrophoresis (PAGE)) and unify the PCR conditions for amplification of any locus.

Table 3:. Nucleotide sequence of primers and characteristics of amplicons for VNTR analysis of brucella

Item	5` -› 3`sequence	Variable region	The length of the amplicon excluding the variable repeats
VBru4F	ctgacgaagggaaggcaa	gtagggca	147 bp
VBru4R	aggcgatctggagattatcg		
VBru6F	gggatgtggtagggtaatcg	ggatgggcagcagacgttacccgcccgcccgcagccgcgaagcgagcgaggacggg-caaaggcaaaagcgggcgtttgcctgggagggcaatgatggcacgcgagcgcatttatatctacgacaccacctcggc	126 bp
VBru6R	gcgtgacaatcgacttttg		
VBru7F	agctgacggggaagaacat	ttccccta	125 bp
VBru7R	ccttttcagtcaaggcaaat		
VBru8F	attattcgcaggctcgtga	tccagcctgtcattctgt	279 bp
VBru8R	caaacagaaggttttccagct		
VBru9F	catggcggattcgttctt	actgccct	103 bp
VBru9R	gggagtatgttttggttgtacatag		
VBru11F	gctgttgatctgaccttgca	actgccattggccagaatcgtacctcgattagtaccaatgccaagggcgttgccgacaacaag	195 bp
VBru11R	ccagacaacaacctacgtcct		
VBru16F	ggagtttttgttgctcaatgtt	aagggagt	132 bp
VBru16R	gccatgtttccgttgatttat		
VBru18F	atgttagggcaatagggcag	gagtaagg	137 bp
VBru18R	gatggttgagagcattgtgaag		
VBru19F	cgacccggaccatgtct	cacgat	161 bp
VBru19R	tcaccgtaacgtcgtggat		
VBru21F	gctcatgcgcaaccaaa	ctattgcc	120 bp
VBru21R	atctcgtggtcgataatctcatt		
VBru30F	tgaccgcaaaaccatatcc	agggcagt	113 bp
VBru30R	aatatgtgcagagcttcatgttc		
VBru42F	tcgcctcaactataccgtca	aagcgccatcatttttcgtgggtgacgatgacggggatcaagagatcaccccagtt-gccgtcgccgttcccgtgttgccgaaggcacacaaagagaagaaacatggcggcgacggcaattgttga	162 bp
VBru42R	accgcaaaatttacgcatc		
VBru43F	tcaagcccgatatggagaat	ccgtttacccgc	152 bp
VBru43R	tccgcctgcccataaac		
VBru45F	tcatccttgcctctccctac	cggcggcggcgcttcggg	116 bp
VBru45R	gggtaaatatcaatggcttgg		
VBru55F	aggctgtttcgtcatgtcttt	gcggcactccacgcctcttggttatatccctgccgtgcct	183 bp
VBru55R	tctggcgttcgagttgttc		

The nucleotide sequence may contain point replacements. The molecular weight of the amplified fragment consists of the length of the amplicon without taking into account the variable repeats and the value of a particular variable region, with the multiplicity represented in the genome of a particular strain. The analysis of the variable amount of such repeats (determination of the number of these repeats), respectively, was carried out by calculating the difference in the molecular weight of the amplicon (after electrophoresis) and the magnitude of the amplified fragment excluding the variable repeats (Table 3), the resulting digit was divided by the number of nucleotides of the variable region (the nucleotide sequence is represented in Table 3); an example of the calculation of the number of variable repeats is presented below. The typical profile of MLVA for some *B. abortus* and *B. melitensis* strains and isolates is presented at: http://vnivi.ru/images/mlva.xls.

A sample of the calculation of the number of variable repeats upon amplification of the "Bru4" locus, if: the molecular weight of the amplicon is 187 bp; the magnitude of the amplified fragment without taking into account the variable repeats 147 bp; nucleotide sequence of the 8 bp variable region is "gtagggca". The number of variable repeats is calculated as follows: 187-147=40, 40/8=5. The number of variable repetitions of the locus "Bru4" is 5.

3.4 Design and Construction of Positive Control

To verify the correctness of the design of the primers and the probe, the correct composition of the reaction mixtures in PCR and the amplification program, a positive control was created, presented as a plasmid DNA with a specific insert for amplification of bacteria of the genus Brucella and determining their belonging to *B. abortus* and *B. melitensis* (complementary developed primers and probes). The nucleotide sequence of the specific insert is an alternation of loci complementary to direct primers for indicating each of the three DNA markers, then alternation of the loci complementary to the probes to indicate each of the three DNA markers and the loci complementary to the reverse primers to indicate each of the three DNA markers. Thus, the original nucleotide sequence of all three marker regions is imitated. The specific insert created had the following nucleotide sequence: "5`tcgaatggctcggttgccagcaatcgtcgtattgccactataatcattgcttggcacctcgg aaacaccgatcaagtcgggcgctctggaccgaaaggatcagcgtgccagaatgaa-gcgctgcagacaaattttgacttccttctggcgacgctttacccgttgttcga-gaactgcgccgtctcaaactctatcggctttcgggaatg3`". The complementarity of the primers developed and the positive control is illustrated in Figure 2.

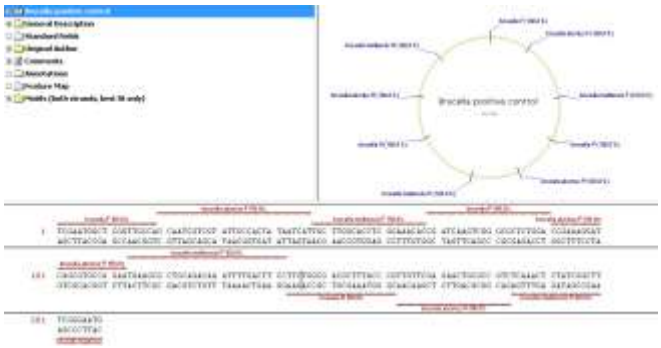


Fig. 2:. Complementarity of primers and probes for the indication of bacteria of the genus Brucella and species *B. abortus* and *B. melitensis* to the nucleotide sequence of the positive control

Synthesis and an insert into the plasmid vector "pAL2-T" were provided by Evrogen, Russia. As a result, PCR with this positive control accumulates amplification products of molecular weight from 162 bp to 164 bp, which allows to control PCR (with any complex of oligonucleotide primers indicated in Table 1) both in electrophoretic format and in real time.

3.5 Polymerase Chain Reaction with Developed Primers and Probes

The performance of all primers and probes is confirmed by amplification. Primers and probes for indicating bacteria of the genus Brucella and determining their belonging to *B. melitensis and B. abortus* were amplified from positive control DNA and from the DNA of vaccine strains of *B. abortus and B. melitensis*; the amplification result is shown in Figure 3.

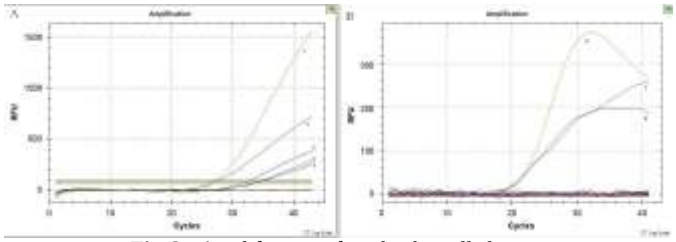


Fig. 3:. Amplification of marker brucella loci

Nomenclature: PCR with DNA matrix of *B. suis, B. abortus* and *B. melitensis* (A); PCR with a positive control DNA matrix at a concentration of 1 μg/μl (B); the PCR result for the Fam channel (2, 3, 5, 7); the PCR result for the Rox channel (1, 6); the PCR result for the R6G channel (4, 8); amplification of *B. suis* DNA (3); amplification of *B. abortus* DNA (1, 2); amplification of *B. melitensis* DNA (4, 5).

The PCR used a mixture of primers and probes to simultaneously show (in one tube) the bacteria of the genus Brucella and determine their belonging to *B. abortus* and *B. melitensis.* As a result of amplification with *B. suis* DNA, PCR was performed only with generic primers and a probe (via the FAM channel). Amplification with the *B. abortus* and *B. melitensis* DNA was successful not only for generic primers, but also for specific primers and probes (via Rox and R6G, respectively). Specific amplification occurred only with the desired DNA. With positive control DNA, amplification was successful in all three channels, which confirms their correct design.

The use of primers for MLVA was performed using a DNA of *B. melitensis* strain Rev.1 as DNA matrix; the amplification results (indicating the molecular weight of amplicons and the number of variable repeats) are shown in Table 4.

Table 4:. Amplification results for MLVA

Locus	Molecular mass of the amplicon	Number of repeats
VBru4	155	1
VBru6	390	2
VBru7	150	3
VBru8	330	3
VBru9	150	6
VBru11	260	1
VBru16	150	2
VBru18	170	4
VBru19	160	0
VBru21	150	4
VBru30	150	5
VBru42	410	3
VBru43	180	2
VBru45	150	2
VBru55	260	2

Comparison of the MLVA profiles of different isolates and strains of brucellas allows us to establish in a relatively short time (2-3 days) the strain belonging to the analyzed species of brucella, and to find identical, similar or different isolates.

4. Conclusion

In the course of the research work the following **conclusions** were drawn:

1. As a result of the bioinformational analysis, DNA marker loci for indicating bacteria of the genus Brucella and determining their belonging to the species *B. abortus* and *B. melitensis* have been determined. The design of primers and probes for qPCR is made, which allows amplifying these loci.
2. Nucleotide sequences for MLVA are adapted for use in the conventional PCR laboratory settings, under the same conditions and modes of research.
3. A unique positive control, containing the nucleotide sequences of the indicated loci, has been created.
4. The described MLVA-technique allows for effective differentiation of different isolates and strains of brucella and their possible belonging to certain strains of brucella from genomic databases.

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