UDC 579.25; 579.26; 579.8:616-036.22 Original articles

THE USE OF MOLECULAR GENETIC METHODS BASED ON MLVA ANALYSIS TO CONFIRM THE UNIQUENESS OF COLLECTION STRAINS OF *BRUCELLA*

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ABSTRACT

Collections of bacterial cultures are used for the storage of industrially valuable and prototypic strains, including the purpose of patenting, manufacturing and testing diagnostic and prophylactic means. The collections are also indispensable to preserve epidemiological strains produced during microbiological tests. Well-prepared collections are suitable for detailed study and tracking of changes accumulating during the evolutionary history of pathogens. Collections are being supplemented with new strains, so it is important that to confirm the uniqueness of these by using highly discriminatory molecular methods. This article presents the results of assessing the genetic diversity of 36 collection strains of *Brucella abortus* and 57 strains of *Brucella melitensis* by Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA). Generic identification and absence of contamination by other bacterial species was confirmed by sequencing of the 16S rRNA gene. The MLVA analysis discovered a contamination of 4 strains with different *Brucella* genotypes and revealed that the deposited 36 strains isolated during 14 brucellosis outbreaks are actually of the same genotype. Our study confirmed the presence of identical strains of *Brucella* in the studied collection. The use of MLVA-16 in testing of bacterial cultures isolated from the same outbreak allowed differentiating unique strains for the subsequent deposition. Validation of the deposited strains with MLVA with detailed analysis of the accompanying metadata allowed optimizing the collection to retain only unique strains, thus reducing economic costs and biosafety risks. The saved funds thus can be used to expand collections with more unique strains.

Keywords: *Brucella melitensis*; *Brucella abortus,* genotyping; MLVA; Kazakhstan.

INTRODUCTION

Bacteria are the most diverse organisms on the planet and have a huge impact on the functioning of ecosystems, including also higher eukaryotic organisms, soils, oceans, as well as diverse bacteria involved in infectious pathologies of animals and plants [1, 2, 3]. The metabolic activity has allowed the use of bacteria in food biotechnology, in the production of fermented beverages and products (cheeses, yoghurts, wines, etc.) even before the isolation of pure bacterial cultures. The development of methodological approaches allowing obtaining, isolating and studying pure bacterial cultures, thus contributing to the rapid development of microbial biotechnology. Currently, bacteria are the main ingredient in the biotechnological production of not only fermented foods, but also alcohol, lactic acid, citric acid, vitamins, amino acids, solvents, antibiotics, biopolymers, biopesticides, industrial enzymes, bio-dyes, biosurfactants, alkaloids, steroids, etc. [4]. Research in the field of biotechnology and bacteriology, published in papers and patents, requires the availability of live bacterial strains in accessible depositories for collecting and independently verifying the results [5]. The preservation of pathogenic bacterial species is necessary for the development of specific means of prevention, diagnostics or as reference cultures to test products [6, 7, 8]. In addition, the study of the properties of isolated pathogens when monitoring territories in the case of natural focal infections and investigating the ways of import and spread of the pathogen in an atypical region is of great importance for ensuring sanitary and epidemiological well-being [9]. Collections and depositories of microorganisms are indispensable for this important function, and are used for depositing, documenting, maintaining, preserving and distributing representative and unique strains [10].

The first collection of bacterial cultures was created by Frantisek Král in 1890 in Prague, and in 1900 he published a catalog. Subsequently, collections of microorganisms began to open in many universities and government organizations, now there are more than 560 collections [11]. Collections of industrially valuable strains and pathogens of infectious diseases are officially registered in the Republic of Kazakhstan. According to the Decree No. 850 of the Government of Kazakhstan (dated July 30, 2002), depositories of pathogens of especially dangerous infections were approved in the RSE «Kazakh Scientific Center for Quarantine and Zoonotic Infections named after Masgut Aikimbayev» and the RSE on REM «National Reference Center for Veterinary Medicine» of the Ministry of Agriculture of the Republic of Kazakhstan (NRTSV). The activities of the depository in the NRTSV are regulated by the Rules for the Deposit of Strains of Microorganisms, Maintaining the National Collection of Deposited Strains of Microorganisms, instilled by Order No. 93 of the Minister of Agriculture of the Republic of Kazakhstan dated March 17, 2020 (hereinafter referred to as the Rules for Depositing). According to which strains of microorganisms existing in Kazakhstan, as well as those obtained as a result of scientific research, in the diagnosis of animal diseases, are subject to storage in the National Collection of Deposited strains of microorganisms used in Veterinary Medicine. Thus, all bacterial strains isolated during the diagnosis of infectious diseases of animals are subject to deposition. At the same time, it should be noted that when diagnosing endemic diseases in Kazakhstan, such as brucellosis, a number of isolated strains from one focus can reach several dozen. When diagnosing anthrax, several samples from a sick animal and environmental objects are analyzed, in which, according to epizootological data, a contamination of *Bacillus an-*

thracis from a fallen or culled animal is possible. In the case of several infected animals, up to 20 bacterial cultures can be isolated, which actually, as a rule, represent one strain. But considering the probability of having several sources of the infection, more strains can be subjected to the collection. With this regard, an excessive storage of large numbers of strains entails a financial burden on the collections, in a form of direct costs associated with maintaining the strains, and also creates additional risks to the personnel. To reduce the costs and the hazard, it is advisable to confirm the uniqueness of the strains isolated during a particular outbreak.

Modern genetic methods make possible to differentiate strains and distinguish outbreaks when caused by leaks from different reservoirs. The methods of analyzing the complete bacterial genome have the highest resolution for genetic variability, but despite the high discriminatory ability, the complete genome sequencing is costly and requests specific skills for complex bioinformatic analysis [12]. Considering a balance between the discriminatory power and resources required it is more appropriate for the initial analysis of strains to use methods based on the analysis of genome fragments, such as Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA) and multilocus sequence typing (MLST). These methods act as the gold standard of genotyping for certain bacterial species (*Brucella spp., Bacillus anthracis, Neisseria meningitidis*) [13]. The purpose of this study is to assess the discriminatory possibility of MLVA in the identification of strains of *Brucella spp.* including those from the same outbreak in a collection of *Brucella* strains of the NRTSV.

MATERIALS AND METHODS

Strains and DNA isolation

Brucella spp. strains were stored in a cryoprotective environment at -80°C. After thawing on ice, the bacterial strain was inoculated onto Brucella Agar (HiMedia), incubated at 37°C in atmosphere of 5-10% CO2 for 5 days with colony growth tracking from day 3. Individual bacterial cultures were dispersed in 0.3 ml of TE buffer (Tris 10 mM EDTA 1 mM pH 8), inactivated by addition of an equal volume of chloroform. DNA isolation was performed by QIAamp DNA Mini Kit (Qiagen, USA). DNA concentration was determined on a BioPhotometer plus (Eppendorf). DNA was stored at -20°C before the study.

Generic identification and evaluation of contamination of samples

In order to confirm the species identification, a 16S rRNA fragment was analyzed. Primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 806R (5'-GGACTACCAGGGTATCTAAT-3') were used [14]. A 25 ul reaction mixture contained 300nM of each primer, 1x PCR Gold Buffer (Applied Biosystems, 4311806), 0.2 mM each dNTP, 1.5 mM MgCl2, 3 ng DNA and 1U AmpliTaq Gold DNA Polymerase. PCR cycling was performed on a Master Cycler Pro S machine (Eppendorf), and included initial denaturation of 10 minutes at 95°C; 30 cycles of 95°C -20 seconds, 55°C – 30 seconds and 72°C for 1 minute; final extension step was 72°C - 5 minutes. Electrophoresis was performed in 1.5% agarose gel, ethidium bromide was used as an intercalating dye. PCR products before sequencing were purified by the enzymatic method using ExoSAP-IT PCR Product Cleanup (Applied Biosystems), according to the manufacturer's instructions. Sequencing was generated using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), followed by separation into ABI Prizm 3730xl. Chromatograms were evaluated and contigs were combined in Lasergene (DNAStar). The species identification was carried out using the BLAST resource (https://blast.ncbi.nlm.nih .gov/).

MLVA genotyping and tree construction

For MLVA genotyping, we used a panel of 16 VNTR loci proposed by Le Flèche et al. and modified by Al Dahouk et al [15, 16]. The panel includes 8 minisatellite VNTR loci (Bruce06, Bruce08, Bruce12, Bruce42, Bruce43, Bruce45 and Bruce55) and 8 microsatellite loci (Bruce18, Bruce19, Bruce21, Bruce04, Bruce07, Bruce09, Bruce16 and Bruce30). Microsatellite loci enable to differentiate species and assess the global distribution of genotypes, while the combination of all loci allows discriminating strains from particular outbreaks. The combination of primers, their concentration and PCR conditions were performed as described earlier [17, 18]. PCR amplification products were diluted 70 times and separated into DNA Analyzer 3730xl (Applied Biosystems, Japan). VNTR repeat size analysis was performed using GeneMapper 4.1 (Applied Biosystems). Dendograms were constructed using the Maxim parsimony analysis method in BioNumerics 7.6 (Applied Maths, Sint-Martens-Latem, Belgium).

RESULTS

A total of 93 brucella strains were studied, including 36 strains of *B. abortus* and 57 strains of *B. melitensis.* Total concentration of the isolated DNA varied from 4 to 8 micrograms per 1 ml. By analyzing the nucleotide sequence of 16S rRNA, all analyzed strains were assigned to *Brucella spp.* with 100% confidence. Sequencing results did not contain additional peaks, thus excluding DNA contamination by other bacterial species.

Values of 16 VNTR loci were obtained for all analyzed strains. A contamination of the profile in hypervariable loci was observed in 4 strains (Figure 1). In the *B. melitensis* strain m54, 5 and 7 alleles were identified at the Bruce 30 locus. In the strain *B. abortus* a 13, at the locus Bruce-07, 5 and 7 alleles were established. In the *B. melitensis* strain m55, the contamination of one loci was observed, specifically at the Bruce-09 locus (alleles 6 and 7). Also, the Bruce-16 locus (alleles 3 and 4) is contaminated in the *B. melitensis* m56 strain.

The studied 35 strains of *B. abortus* formed 14 genotypes (Figure 2). Genotypes 2-6, 9-11 and 13 are represented by only one strain. Genotype 1 comprised 6 strains: 2 strains isolated from cattle in the Issyk settlement in 1961, 1 strain from the Kazakh Institute of Agriculture was collected in the Almaty region, and 3 strains of 2015 were from three settlements of the West Kazakhstan region. Genotype 7 combines 5 strains from cattle in the Kostanay region deposited by the Republican Veterinary Laboratory (RVL) in 2008. Genotype 8 combines 9 strains of which 7 were isolated in the Kostanay region from cattle in 2008 and 2011, and by 1 strain from the Almaty and Atyrau regions. Genotypes 12 and 14 combine 3 strains from the Almaty and West Kazakhstan regions, these strains were isolated in different localities. After analyzing the geographical localization, source and year of isolation, it can be postulated that the strains of the genotype 1 num-

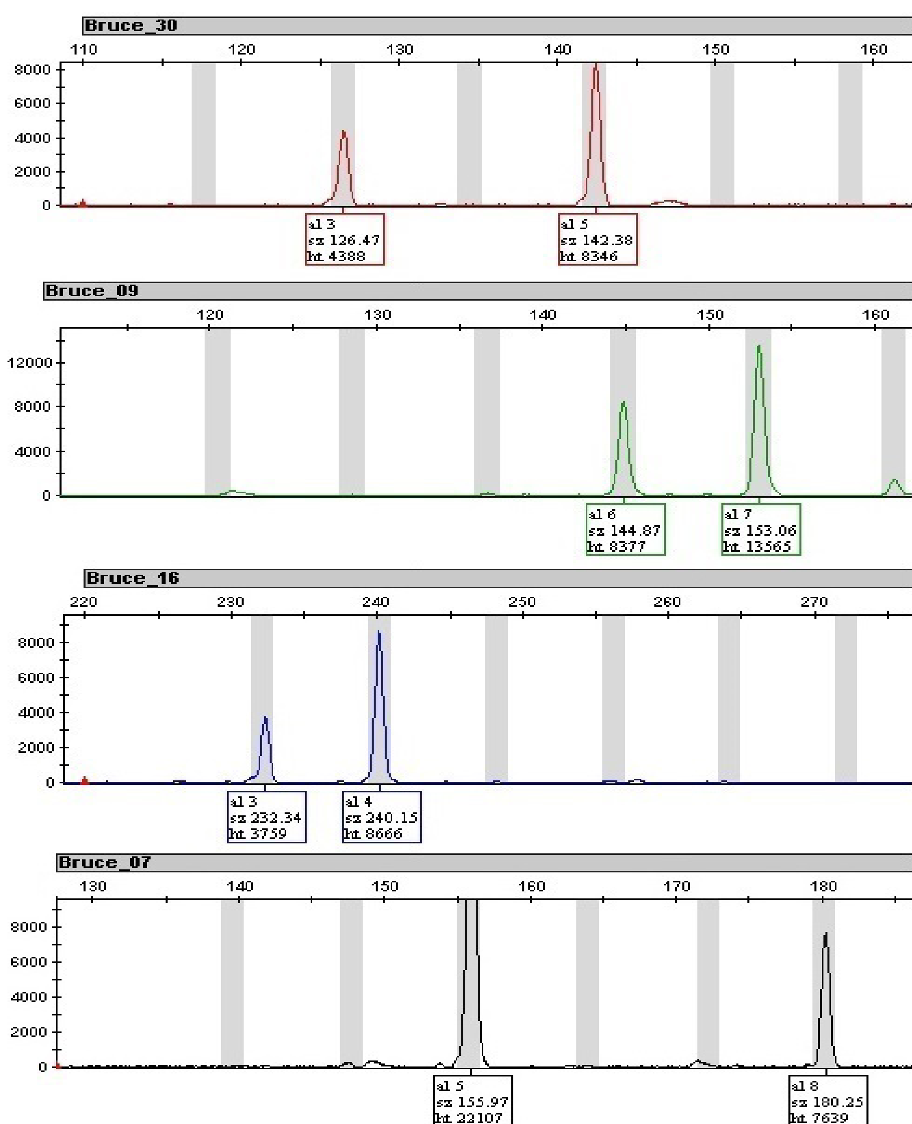

Figure 1 – Electrophoretic profiles of contaminated samples

bered a1 and a2 are identical and represent the same outbreak. The strains a17, a19, a20, a22 and a23 of genotype 7 deposited by RVL may represent actually one strain isolated during one outbreak in the Kostanay region. The strains a16, a18 and a21 may be further revised as being the same strain. For the strains a25, a26 and a27, more data on the isolation site and depositor are required.

Among studied *B. melitensis* cultures with pure MLVA profiles, 53 strains were clustered into 14 genotypes (Figure 2). Genotypes 18, 20, 22, 25-29 are represented by single strains. The largest genotype 16 combined 22 strains: 19 strains from 11 settlements of the Zhambyl region, isolated in 2008, 2012 and 2013; 2 strains from 2 settlements of the West Kazakhstan region; 1 strain isolated in 2013 in the Almaty region. Genotype 17 combines 6 strains from 5 settlements of the Zhambyl region and 1 strain from the Almaty region. Genotype 19 combines 6 strains from small cattle in the settlement of Karaturuk in the Almaty region and 1 strain from the Almaty region without specifying the exact geographical location. Genotype 23 combined 2 strains isolated in 2013 from small cattle in the village of Bereke in the Almaty region. Genotype 24 combined 4 strains, two of which were isolated in the settlement of Koktal and by 1 strain from the settlements of Ushkonyr and Karaoi of the Almaty region. Taking into account metadata for the samples of genotype 16, the identity of the following strains can be postulated: m23 and m44; m26, m49 and m51; m28 and m29; m32 and m33; m36 and m37; m45, m46 and m47. In genotype 17, the strains m24 and m39 are identical. Genotype 19 appears to be a separate strain from a single outbreak, with the exception of the poorly characterized strain m15. Two strains of genotype 23 are also identical. In the genotype 24, the strains m6 and m19 isolated from small cattle in the settlement of Koktal in 2013, are genetically homogeneous and apparently represent the same outbreak.

DISCUSSION

Microorganism collections, while performing their main functions of depositing for the purposes of patent procedure and maintaining biological diversity, hold representative samples of microbial diversity that cover a large number of species [19]. With this regard, the collections of bacterial pathogens need to expand their taxonomic representation, as well as track and deposit strains with intraspecific differences. The latter are needed to study evolution of circulating strains and respond timely to the ongoing pathogen adaptation [20, 21]. However, depositing every isolated strain from an outbreak results in a quick increase in a number of individual sam-

Key	Bruce_06	Bruce_08	Bruce_11	Bruce_12	Bruce_42	Bruce_43	Bruce_45	Bruce_55	Bruce_18	Bruce_19	Bruce_21	Bruce_04	Bruce_07	Bruce_09	Bruce_16	Bruce_30	Species	Host	Year	Region	Village	Genotype
a1	4	5	3	12	2	2	3	1	6	43	8	4	7	6	3	3	B. abortus	cattle	1961	Almaty	Issyk	1
a2	4	5	3	12	2	2	3	1	6	43	8	4	7	6	3	3	B. abortus	cattle	1961	Almaty	Issyk	
a6	4	5	3	12	2	2	3	1	6	43	8	4	7	6	3	3	B. abortus	cattle	1967	Almaty	KIZ	
a30	4	5	3	12	2	2	3	1	6	43	8	4	7	6	3	3	B. abortus	cattle	2015	West-Kazakhstan	Kyzyloba	
a35	4	5	3	12	2	2	3	1	6	43	8	4	7	6	3	3	B. abortus	cattle	2015	West-Kazakhstan	Merey	
a36	4	5	3	12	2	2	3	1	6	43	8	4	7	6	3	3	B. abortus	cattle	2015	West-Kazakhstan	Zhangala	
a12	4	5	3	12	2	2	3	1	6	45	8	4	7	6	3	3	B. abortus	cattle	1962	East Kazakhstan	Bukhtarma	2
a3	4	5	3	12	2	2	3	1	6	43	8	4	5	7	3	3	B. abortus	sheep	1960	Almaty		3
a4	4	5	3	12	2	2	3	1	6	43	8	5	6	6	3	3	B. abortus	cattle	1960	Almaty	Kirov	4
a11	4	5	3	12	2	2	3	1	6	43	8	5	6	11	3	3	B. abortus	cattle	1970	Aqmola	Hleborob	5
a15	4	5	3	12	2	2	3	1	6	43	8	5	6	7	3	3	B. abortus	camal	1969	Atyrau	Balkuduk	6
a17	4	5	3	12	2	2	3	1	6	43	8	5	7	9	3	3	B. abortus	cattle	2008	Kostanay	Republican Veterinar	7
a19	4	5	3	12	2	2	3	1	6	43	8	5	7	9	3	3	B. abortus	cattle	2008	Kostanay	Republican Veterinar	
a20	4	5	3	12	2	2	3	1	6	43	8	5	7	9	3	3	B. abortus	cattle	2008	Kostanay	Republican Veterinar	
a22	4	5	3	12	2	2	3	1	6	43	8	5	7	9	3	3	B. abortus	cattle	2008	Kostanay	Republican Veterinar	
a23	4	5	3	12	2	2	3	1	6	43	8	5	7	9	3	3	B. abortus	cattle	2008	Kostanay	Republican Veterinar	
a9	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	1952	Almaty		8
a14	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	1968	Atyrau	Tendyk	
a16	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	2008	Kostanay	Republican Veterinar	
a18	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	2008	Kostanay	Republican Veterinar	
a21	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	2008	Kostanay	Republican Veterinar	
a24	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	2011	Kostanay		
a25	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	2008	Kostanay		
a26	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	2008	Kostanay		
a27	4	5	3	12	2	2	3	1	6	43	8	5	7	10	3	3	B. abortus	cattle	2008	Kostanay		
a5	4	5	3	12	2	2	3	1	6	43	8	5	7	5	3	3	B. abortus	cattle	1957	Almaty	Almaty biokombinat	9
a29	4	5	3	12	2	2	3	1	6	43	8	5	7	6	3	3	B. abortus	cattle	1969	West-Kazakhstan	Chapaev	10
a33	4	6	3	12	2	2	4	1	6	49	8	4	7	6	3	6	B. abortus	cattle	2015	West-Kazakhstan	Merey	11
a7	4	5	4	12	2	2	3	3	6	43	8	3	5	3	3	5	B. abortus	cattle	1947	Almaty	OLB 9	12
a10	4	5	4	12	2	2	3	3	6	43	8	3	5	3	3	5	B. abortus	cattle	1953	Almaty		
a28	4	5	4	12	2	2	3	3	6	43	8	3	5	3	3	5	B. abortus	cattle	1969	West-Kazakhstan	Chapaev	
a31	4	5	4	12	2	2	3	3	5	43	8	3	5	3	4	5	B. abortus	cattle	2015	West-Kazakhstan	Saihin	13
a8	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	5	B. abortus	cattle	1953	Almaty		14
a32	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	5	B. abortus	cattle	2015	West-Kazakhstan	Merey	
a34	4	5	4	12	2	2	3	3	6	43	8	4	4	3	4	5	B. abortus	cattle	2015	West-Kazakhstan	Mashketsay	
m44	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Kassyk	15
m23	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Kassyk	
m52	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2015	West-Kazakhstan	Koskul	
m1	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2008	Jambyl	Zhalpak tobe	16
m27	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Korday	
m28	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Otar	
m29	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Otar	
m30	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Nogaibai	
m32	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Akkum	
m33	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Akkum	
m35	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Bostandy	
m36	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Tamdy	
m37	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Tamdy	
m42	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2012	Jambyl	Zhana zhol	
m17	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Almaty	Kainar	
m45	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Karasay	
m46	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Karasay	
m47	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Karasay	
m49	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Turar Ryskulov	
m51	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Turar Ryskulov	
m26	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2013	Jambyl	Turar Ryskulov	
m53	1	5	3	13	2	2	3	2	4	41	8	5	4	3	6	5	B. melitensis	sheep	2015	West-Kazakhstan	Merey	
m4	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	B. melitensis	sheep	2013	Almaty	Bakanas	17
m24	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	B. melitensis	sheep	2013	Jambyl	Kenes	
m25	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	B. melitensis	sheep	2013	Jambyl	Birlik	
m39	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	B. melitensis	sheep	2013	Jambyl	Kenes	
m43	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	B. melitensis	sheep	2013	Jambyl	Kassyk	
m48	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	B. melitensis	sheep	2013	Jambyl	Zhambyl	
m50	1	5	3	13	2	2	3	2	4	41	8	5	4	3	4	5	B. melitensis	sheep	2013	Jambyl	Turar Ryskulov	
m20	1	5	3	13	2	2	3	2	4	41	8	4	4	3	6	5	B. melitensis	sheep	2011	Almaty	Karaturuk	18
m10	1	5	3	13	2	2	3	2	4	41	8	7	4	3	6	6	B. melitensis	sheep	2011	Almaty	Karaturuk	19
m11	1	5	3	13	2	2	3	2	4	41	8	7	4	3	6	6	B. melitensis	sheep	2011	Almaty	Karaturuk	
m12	1	5	3	13	2	2	3	2	4	41	8	7	4	3	6	6	B. melitensis	sheep	2011	Almaty	Karaturuk	
m13	1	5	3	13	2	2	3	2	4	41	8	7	4	3	6	6	B. melitensis	sheep	2011	Almaty	Karaturuk	
m14	1	5	3	13	2	2	3	2	4	41	8	7	4	3	6	6	B. melitensis	sheep	2011	Almaty	Karaturuk	
m15	1	5	3	13	2	2	3	2	4	41	8	7	4	3	6	6	B. melitensis	sheep	2011	Almaty		
m22	1	5	3	13	2	2	3	2	4	41	8	7	4	3	6	6	B. melitensis	sheep	2011	Almaty	Karaturuk	
m3	1	5	3	13	2	2	3	2	4	41	8	7	4	3	6	5	B. melitensis	sheep	2013	Almaty	Bakanas	20
m9	1	5	3	13	2	2	3	2	4	41	8	11	4	3	7	6	B. melitensis	sheep	2011	Almaty	Karaturuk	21
m31	1	5	3	13	2	2	3	2	4	41	8	11	4	3	7	6	B. melitensis	sheep	2013	Jambyl	Nogaibai	
m16	1	5	3	13	2	2	3	2	4	41	8	4	4	3	7	6	B. melitensis	sheep	2013	Almaty	Kainar	22
m5	1	5	3	13	2	2	3	2	4	41	8	4	4	3	4	6	B. melitensis	sheep	2013	Almaty	Bereke	23
m18	1	5	3	13	2	2	3	2	4	41	8	4	4	3	4	6	B. melitensis	sheep	2013	Almaty	Bereke	
m6	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	6	B. melitensis	sheep	2013	Almaty	Koktal	24
m7	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	6	B. melitensis	sheep	2013	Almaty	Ushkonyr	
m8	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	6	B. melitensis	sheep	2013	Almaty	Karaoi	
m19	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	6	B. melitensis	sheep	2013	Almaty	Koktal	
m40	1	5	3	13	2	2	3	2	4	41	8	5	4	3	5	6	B. melitensis	sheep	2012	Jambyl	Baluan Sholak	
m41	1	5	3	13	2	2	3	2	4	41	8	6	4	3	8	6	B. melitensis	sheep	2012	Jambyl	Zhana zhol	25
m2	1	5	3	13	2	2	3	2	4	41	8	4	4	3	7	7	B. melitensis	sheep	2012	Almaty	Karazhota	26
m21	1	5	3	13	2	2	3	2	4	41	8	4	4	3	6	7	B. melitensis	sheep	2011	Almaty	Karaturuk	27
m34	1	5	3	13	2	3	3	2	4	41	8	7	4	3	7	5	B. melitensis	sheep	2013	Jambyl	Bostandy	28
m38	1	5	3	13	2	3	3	2	4	41	8	5	4	3	8	5	B. melitensis	sheep	2013	Jambyl	Zhambyl	29

Figure 2 – Dendrogram based on the MLVA-16 genotyping assay (UPGMA method)

ples, which may skew the species diversity in the collection and will require additional resources to maintain the collection. In order to keep a balance between preserving biological diversity in the collection and excluding identical strains, depositors must use highly discriminatory methods to distinguish identical strains isolated from the same outbreak. This pilot study was aimed at assessing the genetic diversity in the collection of *Brucella spp*. using MLVA and identifying identical strains from the same outbreaks. Based on the MLVA genotyping and metadata, the presence of 36 strains originating from 14 outbreaks with the identical genotypes was established.

The MLVA genotyping using 16 VNTR loci has proven to be a highly discriminatory method allowing clustering strains by geographical origin, as well as combining strains isolated from different patients within the same outbreak or from the same patient before first-line therapy and after relapse [22].

The method has high reproducibility, and the markers used remain stable, which has been proven during genotyping of *B. melitensis* strains Rev-1 isolated from humans and animals from different geographical regions, as well as during genotyping of 40 passages of the *Brucella suis* vaccine strain S2 [23]. With this regard, MLVA-16 can be considered as the primary test of identifying strains from the same outbreak. Nevertheless, to confirm the identity of the strains, it is necessary to also analyze metadata which allows comparing the strains by a place and time of isolation. Since microsatellites are prone to homoplasia, so that a same genotype can occur in unrelated samples isolated in different time periods and different regions [24]. Exemplary, in our study, the lack of matadata on the m15 strain did not allowed to evidence that this strain is from the same outbreak as the other strains m10-m14 and m22.

The MLVA genotyping revealed a high level of homoplasia among the analyzed strains, as only 14 genotypes were identified among 35 strains of *B. abortus* and 53 strains of *B. melitensis*. The largest genotype in *B. melitensis* strains combined 22 strains collected on the territory of 3 regions in 2008-2015. However, uncontrolled movement of animals can also result in the wider geographical presence of one strain, which was demonstrated in the work of A. Daugalieva [25]. Actually, a presence of an identical MLVA profile has been described in unrelated strains, which justifies genome-wide analysis for further association of strains with an outbreak [26].

The MLVA genotyping using hypervariable microsatellite loci can be used to check the stability of strains during long storage, since MLVA allows detecting small genomic variations. This was demonstrated by testing 63 batches of commercial vaccines RB51 and S19 produced by nine different manufacturers. In two batches, a difference was found from the typical strains at the Bruce-07 locus [27].

CONCLUSION

Controlling for the representation in a bacterial collection of unique strains increases the value and significance of the collection from a scientific and epidemiological point of view. TRhe presented study has demonstrated the presence of strains with identical MLVA-16 profile, and isolated in the same territory during same outbreak, in the NRTSV collection. The analysis provided proofs that some strains in the collection are actually genetically identical. In our opinion, maintaining identical strains in a collection is impractical from an economic point of view and because of posing additional biological hazards. Thus the available collections need to undergo a genetic check in order to maintain and store only unique strains. Only isolated DNA from supposedly the same strains can be stored in place of live bacteria. Following this proposal will reduce costs of maintaining microbiological collections and save reserves for replenishing the collections with other unique genotypes.

FUNDING

The study was carried out with the financial support of the Ministry of Education and Science of the Republic of Kazakhstan within the framework of project AP08052352 for 2020-2022.

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ИСПОЛЬЗОВАНИЕ МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКИХ МЕТОДОВ НА ОСНОВЕ MLVA-АНАЛИЗА ДЛЯ ПОДТВЕРЖДЕНИЯ УНИКАЛЬНОСТИ КОЛЛЕКЦИОННЫХ ШТАММОВ БРУЦЕЛЛ

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АБСТРАКТ

Коллекции бактериальных культур необходимы для хранения производственно ценных и типовых штаммов с целью патентования, производства и тестирования диагностических и профилактических препаратов. Коллекции нужны также для хранения штаммов, выделенных в ходе диагностических процедур. Хорошо подготовленные коллекции пригодны для детального изучения и отслеживания изменений, накапливающихся в ходе эволюционной истории патогенов. Коллекции пополняются новыми уникальными штаммами, поэтому очень важно, чтобы изучение характеристик этих штаммов проводилось с использованием высоко дискриминационных молекулярно-генетических методов. В данной статье приведены результаты оценки генетического разнообразия 36 коллекционных штаммов *Brucella abortus* и 57 штаммов *Brucella melitensis* методом мультилокусного анализа тандемных повторов (VNTR MLVA-16). Родовая идентификация и отсутствие контаминации другими видами бактерий была подтверждена секвенированием гена 16S rRNA. MLVA анализ позволил установить контаминацию 4 штаммов разными генотипами бруцелл и выявить, что депонированнные 36 штаммов, выделенные при 14 вспышках бруцеллеза являются идентичными генотипами возбудителя. Наши исследования подтвердили наличие в коллекции идентичных штаммов бруцелл. Использование MLVA-16 в тестировании выделенных бактериальных культур из одной и той же вспышки позволило дифференцировать уникальные штаммы для последующего их депонирования. Проверка депонированных штаммов с MLVA характеристиками с детальным анализом метаданных о депонированных штаммах позволяет оптимизировать коллекцию, для сохранения только уникальных штаммов, что снижает экономические издержки и риски биологической безопасности. Высвободившиеся средства могут быть использованы для пополнения коллекции уникальными штаммами.

Ключевые слова: *Brucella melitensis, Brucella abortus*, генотипирование, MLVA, Казахстан.

БРУЦЕЛЛА КОЛЛЕКЦИЯЛЫҚ ШТАМДАРЫНЫҢ ЕРЕКШЕЛІГІН РАСТАУ ҮШІН MLVA ТАЛДАУ НЕГІЗІНДЕГІ МОЛЕКУЛАЛЫҚ ГЕНЕТИКАЛЫҚ ӘДІСТЕРДІ ҚОЛДАНУ

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ТҮЙІН

Патогенді бактериялар культураларының топтамалары диагностикалық және профилактикалық препараттарды патенттеу, өндіру және сынау мақсатында, сондай-ақ жұқпалы ауруларды диагностикалау кезінде оқшауланған эпидемиологиялық штаммдарды сақтау үшін өнеркәсіптік құнды және типті штаммдарды сақтау үшін қажет. Бұл санитарлық-эпидемиологиялық салауаттылықтағы өзгерістерді егжей-тегжейлі зерттеу және қадағалау үшін жасалады. Топтамалар жаңа, бірегей штаммдармен толықтырылады, сондықтан бұл штаммдардың сипаттамаларын зерттеу, жоғары дискриминацияланған молекулалық-генетикалық әдістерді қолдану арқылы жүзеге асыру өте маңызды. Бұл мақалада Multilocus variable number tandem repeat (VNTR) analysis (MLVA-16) көмегімен Brucella abortus 36 коллекциялық штаммының және Brucella melitensis 57 штаммының генетикалық әртүрлілігін бағалау нәтижелері берілген. Жалпы идентификация және басқа бактерия түрлерімен ластанудың жоқтығы 16S рРНҚ генін секвенирлеу арқылы расталды. MLVA талдауы бруцелланың әртүрлі генотиптерімен 4 штаммның ластануын анықтауға және бруцеллездің 14 ошағы кезінде оқшауланған тұндырылған 36 штаммдардың бірдей патогендік генотиптер екенін анықтауға мүмкіндік берді. Біздің зерттеулер топтамада бруцелланың бірдей штаммдарының болуын растады. MLVA-16-ны бір ошақтан оқшауланған бактерия культураларын сынау үшін қолдану, бірегей штаммдарды саралауға мүмкіндік береді. Депонирленген штаммдардың метадеректерін егжей-тегжейлі талдау арқылы MLVA сипаттамалары бар депонирленген штаммдарды тексеру биологиялық қауіпсіздікті қамтамасыз ету үшін экономикалық шығындар мен тәуекелдерді азайтуға мүмкіндік беретін бірегей штаммдарды сақтау тұрғысынан топтамны оңтайландыруға мүмкіндік береді. Шығарылған қаражатты түр ішілік және түр аралық әртүрлілігімен ерекшеленетін топтамалық штаммдардың бірегей генотиптерін сақтауға пайдалануға болады.

Кілт сөздер: *Brucella melitensis*, *Brucella abortus* генотиптеу, MLVA, Қазақстан.