

GENETIC DIVERSITY OF *B. MELITENSIS* STRAINS ISOLATED IN THE ZHAMBYL PROVINCE OF KAZAKHSTAN DURING 2016-2019

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Abstract

Brucellosis is a dangerous zoonotic infection which also infects humans. Brucellosis-causing pathogen is prevalent throughout the world and causes damage to animal husbandry and public health. Genetic typing methods have become an integral component of the epidemiological/epidemiological surveillance to track the spread of the brucellosis pathogen, because genetic methods allow identifying circulating strains by their unique characteristics. The positive effect of the use of

highly discriminatory genotyping methods was demonstrated on various samples of pathogen's isolates. However, genetic diversity can change over time, and data on the temporal dynamics of the genetic diversity are of interest to study pathogen evolution. In this study, we performed MLVA16 genotyping of *B. melitensis* strains isolated in 2016 and 2019 in the Zhambyl province of Kazakhstan. The results obtained indicate a significant population dynamics of the pathogen, because only 19% of the strains from 2016 had homoplastic analogues among the strains from 2019. Expanding the sample size and observation time are needed to discern reliable picture of the population dynamics and changes in genetic diversity of *Brucella* strains.

Key words: *Brucella melitensis*; genotyping; MLVA; Kazakhstan; cluster analysis.

Introduction

Brucellosis is a zoonotic infection caused by bacteria in the genus *Brucella*, which is prevalent on all continents except Antarctica. [1]. Developing molecular genetics methods for the diagnosis and identification of microorganisms allowed describing new types of *Brucella* and expanded known ranges of the species diversity in the genus, as well as variety of natural hosts of the pathogen [2]. It is currently known, that main economic losses to agriculture and healthcare are caused by three species, *Brucella melitensis*, *B. abortus*, and *B. suis* [3].

The largest incidence of brucellosis infection has been registered in developing countries, where seroprevalence in the population can reach 12%, and even more in risk groups, up to 58% [4, 5]. In Kazakhstan, brucellosis remains among the major veterinary and healthcare problems, with an annual economic burden of USD 24 million to perform routine diagnosing of the disease. [6]. Control strategies currently in effect resulted in a reduction in the registration of new cases of human brucellosis from 23.7

(2004) to 2.8 (2020) per 100 thousand populations and the confirmation of *Brucella* reservoirs in the country.

The danger of brucellosis, importance of the disease control and a possibility of introducing the pathogen into brucellosis-free countries, all requested the development and implementation of genetic fingerprinting methods for use in epidemiological monitoring. The *Brucella* genus is highly homogeneous at the genetic level, with a DNA hybridization rate of 96% ($\pm 5\%$) with ΔT_m less than 1°C among six classic *Brucella* species (*B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*) [7]. This allows stating that the *Brucella* genus is monophyletic and it has been considered as such for long time [8]. Due to the high genetic homogeneity, methods based on the analysis of variable tandem repeats (MLVA) and genome-wide analysis of nucleotide polymorphisms have the greatest discriminatory power [9]. Two main genotyping schemes have been proposed for MLVA genotyping of this pathogen: *Brucella* 'HOOFprints'

[10] and MLVA16 [11, 12]. *Brucella* 'HOOF-Prints' is a panel of 8 microsatellite repeats in which a monomeric unit is AGGGCAGT. MLVA16 allows simultaneous analysis of 16 loci, of which 8 are minisatellite tandem repeats with repeat sizes from 12 to 134 bp (panel 1) and eight microsatellite loci with tandem repeat sizes from 3 to 8 bp (panel 2). Both panels are considered to have sufficient discriminatory ability. However, MLVA16 is more often used because of the higher information content in minisatellite and microsatellite loci, allowing describing the diversity at the global

Materials and methods

B. melitensis strains were collected as part of diagnostic studies in accordance with the current regulations in the Republic of Kazakhstan. The collected strains were ciphered to render them anonymous and sent to the National Scientific Center for Especially Dangerous Infections named after Masgut Aikimbaev of the Ministry of Health of the Republic of Kazakhstan. In connection with the use of anonymous strains obtained by standard research procedures, the conclusion of the ethical commission was not required.

DNA preparation and quality assessment

DNA was isolated from the chloroform-inactivated bacterial biomass using a commercial QIAamp DNA Mini Kit (Qiagen, USA). 16S rRNA sequencing was used for generic identification and exclusion of DNA contamination by several bacterial species according to the protocol proposed by Vegas E.Z.S.

and local levels. In general, MLVA typing allows determining the geographical distribution of isolates, tracing the source and spreading of brucellosis infection, and defining the relationship between strains isolated from animals and humans [13]. However, there is little information in the literature on the stability or changes in the distribution of *Brucella* genotypes over time. With this regard, the purpose of our work was to determine the genetic distribution of *Brucella* genotypes with an interval of collection of biological material over three years in the Zhambyl province of Kazakhstan.

[14]. Sequencing reaction was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoScientific) according to the manufacturer's instructions, followed by fragment separation on an automatic 3730xl DNA Analyzer (Applied Biosystems). Quantification of DNA was performed by a spectrophotometric method using NanoDrop 1000. Additional confirmation of species identification was performed by multiplex PCR [15].

MLVA genotyping of *B. melitensis*

The MLVA16 scheme was used for genotyping. Primers for multiplex PCR and their combinations were used as described earlier [16, 17]. The amplified PCR products were diluted 70 times and 3 µl were used for fragment analysis on an automatic genetic analyzer (DNA Analyzer 3730xl, Applied Biosystems, Japan) in the presence of a LIZ 1200 size standard. VNTR repeat size analysis

was performed using the GeneMapper 4.1 software (Applied Biosystems). To normalize the results of electrophoretic separations, control DNA samples of vaccine strains *B.abortus* RB51, *B. abortus* RS82, and *B. abortus* S19 were used on each plate. To visualize clustering relationships, MST trees were constructed using BioNumerics 8.0

(Applied Maths, Sint-Martens-Latem, Belgium). The Hunter-Gaston Diversity Index (HGDI) was used to describe the discriminating power of each locus as well as the MLVA16 panel (Hunter and Gaston, 1988). The calculations were carried out using the Internet resource http://insilico.ehu.es/mini_tools/discriminatory_power/index.php.

Results

The analysis included 167 strains of *B. melitensis* isolated in the Zhambyl province in 2016, the genotypes of which were previously described in our article [18], as well as 81 strains isolated in 2019 and genotyped in this study. Sizes of 16 VNTR loci were obtained for each strain. The discriminating power (HGDI) of 16 VNTR loci and the MLVA16 panel is shown in table 1.

Table 1 - Discriminating power expressed as Hunter-Gaston Diversity Index (HGDI) of 16 VNTR loci and the MLVA16 panel

Name of loci /panel	Zhambyl 2016		Zhambyl 2019	
	Quantity of alleles/genotypes	HGDI	Quantity of alleles/genotypes	HGDI
Bruce 06	1	0	1	0
Bruce 08	2	0,012	1	0
Bruce 11	1	0	1	0
Bruce 12	1	0	1	0
Bruce 42	1	0	1	0
Bruce 43	3	0,1754	4	0,2244
Bruce 45	1	0	1	0
Bruce 55	1	0	1	0
Bruce 18	2	0,0355	2	0,0488
Bruce 19	4	0,1257	3	0,1404
Bruce 21	1	0	1	0
Bruce 04	8	0,8056	5	0,742
Bruce 07	4	0,1478	3	0,2056
Bruce 09	1	0	1	0
Bruce 16	8	0,8297	9	0,8117
Bruce 30	5	0,6031	7	0,6503
MLVA16	83	0,9818	50	0,9818

The loci Bruce 06, Bruce 11, Bruce 12, Bruce 42, Bruce 45, Bruce

55 Bruce 09 did not have the discriminatory ability in both groups

of strains, and the Bruce 08 locus had only one allele among the strains isolated in 2019. Despite the fact that the sample of strains from 2019 is twice smaller than the sample from 2016, the genetic diversity of the strains is comparable; moreover, the strains' genetic diversity in 2019 exceeds that of 2016 in particular loci, as evidenced by higher HGDI values in the loci Bruce 43, Bruce 18, Bruce 19, Bruce 07, Bruce 30. The discriminating power of all 16 VNTR loci of the MLVA16 panel was 0.9818 in the two groups of strains.

Clustering of *B. melitensis* strains in 2016 and 2019 years

MLVA16 grouped 167 strains of *B. melitensis* 2016 into 83 genotypes, of which 43 are represented by 1 strain, 19 genotypes by 2 strains. 81 strains of 2019 were clustered into 50 genotypes, of which 31 are represented by 1 strain, 13 genotypes by 2 strains (figure 1A). In the 2016 sample, 10 genotypes were identified that combined four or more

strains, of which the largest had 12 strains. In 2019, four genotypes were represented by 4 or more strains, of which the largest has 7 strains. Of the 83 genotypes identified in 2016, homoplasmic analogs among 2019-strains were identified for only 16 (19.3%) genotypes. In a total number of five genotypes from 2016, four or more strains have genetic analogs in strains isolated in 2019, and the largest genotype of 2019 is unique in its genetic profile.

The unique genotypes isolated in 2016 and 2019 combine strains from several regions and are isolated in unrelated outbreaks (figure 1B). For example, one genotype with 11 strains isolated in 2016 was found in four administrative districts (Shuskiy, Zhualinsky, Moyynkumskiy, and Bayzakskiy) and the city of Taraz. An interesting fact is that 80% (4/5) large homologous genotypes from years 2016 and 2019 had been isolated in different geographical locations.

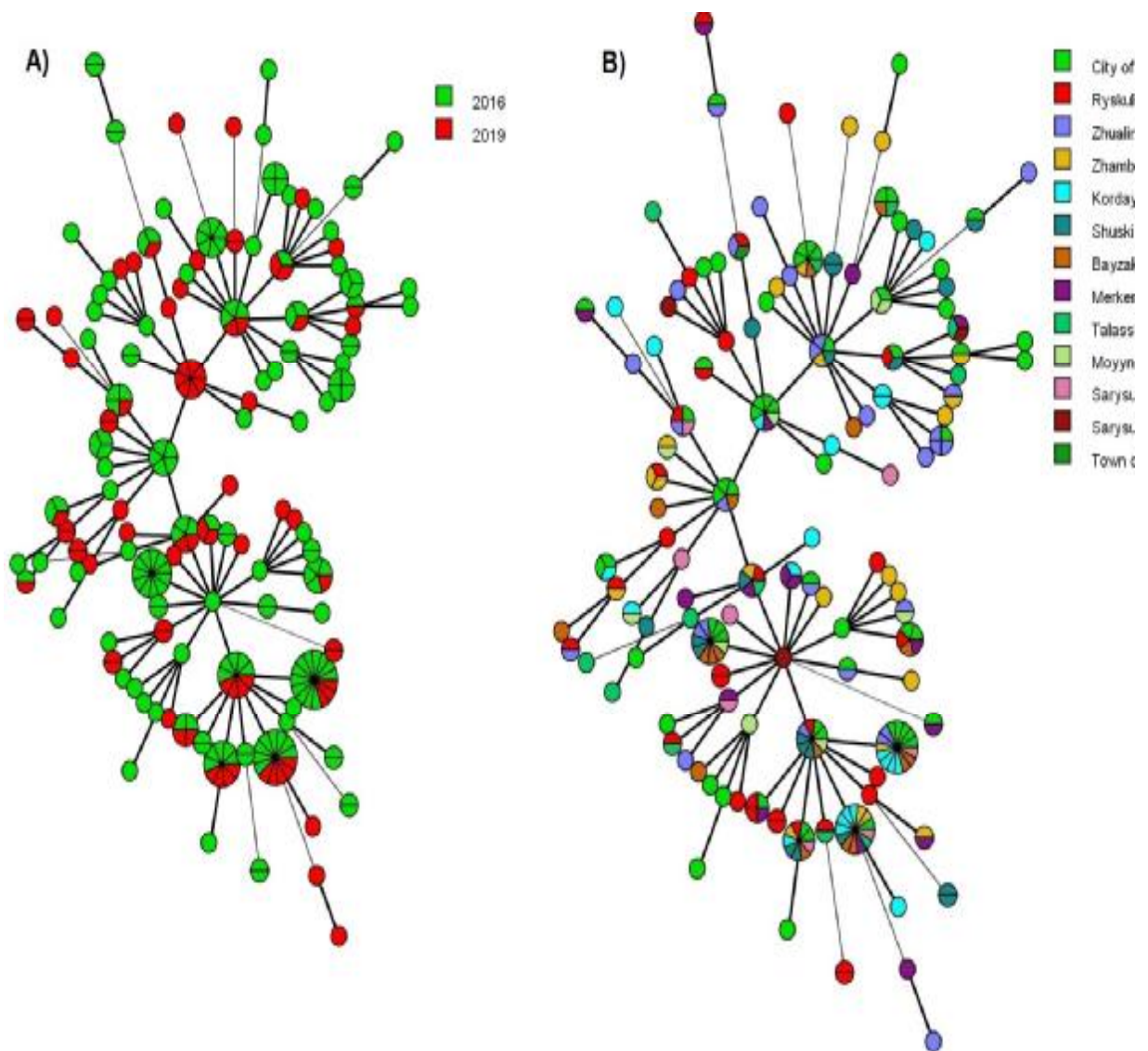


Figure 1 - Maximum parsimony analysis using MLVA16 data from 248 *B. melitensis* strains isolated from humans in 2016 and 2019 years. A - color coding indicates the year of isolation, B - color coding by locations of isolation.

Discussion.

Brucellosis is a classic zoonotic infection in which animals are the main hosts and reservoirs, and serve as sources of infection in people. The infection is transmitted to people through direct contact or consumption of poorly processed foods. Humans are dead-end hosts for *Brucella* because no transmission is possible from the diseased to healthy [19]. In Kazakhstan, the formal confirmation of the diagnosis of brucellosis in animals does not require isolating of

pure culture, but the isolation of pure culture is a standard procedure during the diagnosis of brucellosis in humans. With this regard, the quality of available collections of *Brucella* strains from animals is significantly inferior when compared to collections of *Brucella* strains from humans. We used the *B. melitensis* strains isolated from humans in our study. However, considering the principally zoonotic nature of this infection, our data on the genetic diversity with high

confidence reflect the diversity of *Brucella* in the animal population.

MLVA analysis of *B. melitensis* strains from the Zhambyl province in 2016 and 2019 has a high discriminatory ability at the level of 0.9818. The obtained HGDI value exceeds the threshold of 0.95, which indicates reaching a probability of $\geq 95\%$ for assigning of any two randomly selected unrelated isolates in separate clusters [20]. Earlier, similar results had been obtained for strains isolated in the territory of Kazakhstan, underscoring the high genetic diversity of *Brucella* strains and high discriminatory power of MLVA [21, 22]. The discriminatory power of the Bruce 43 minisatellite locus was higher among strains isolated in 2019. Using minisatellite markers allowed differentiating species and subspecies in the *Brucella* genus. In China, a significant increase for HGDI at the Bruce 43 locus was observed during genotyping a collection of *B. melitensis* bv3 and

bv1 in comparison with the genotyping of a collection represented exclusively by bv3. [23, 24]. Previously, the presence of three biovariants of *B. melitensis* was reported in Kazakhstan [18]. The HGDI of the remaining loci was comparable among the strains isolated in 2016 and 2019.

Our comparative analysis shows a low percentage of genotypic identity among *Brucella* strains from 2016 and 2019. Only 19% of the 2016 genotypes had homoplastic analogs in the 2019 strains. In addition, in the majority of cases, homoplasia was found in various districts of the Zhambyl province. The data obtained indicate a constant change in the population structure of circulating genotypes. One of the possible reasons for the changes in particular areas is the anti-brucellosis measures which are undertaken to curb outbreaks.

Conclusions

Our study confirmed the perseverance of the high genetic diversity among *B. melitensis* strains in Kazakhstan. The obtained results also reveal temporal changes in the majority of circulating *Brucella* strains, however more detailed picture of the evolution in the population structure requests an increase in the sample size and observation time.

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**ГЕНЕТИЧЕСКОЕ РАЗНООБРАЗИЕ ШТАММОВ *B. MELITENSIS*,
ВЫДЕЛЕННЫХ В ЖАМБЫЛСКОЙ ОБЛАСТИ КАЗАХСТАНА ЗА
2016-2019 ГОДЫ**

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Аннотация

Бруцеллез – опасная зоонозная инфекция, способная поражать человека. Возбудитель бруцеллеза широко распространен во всем мире и наносит ущерб животноводству и здоровью населения. Методы генетического типирования стали неотъемлемым компонентом эпидемиологического надзора за распространением возбудителя бруцеллеза, поскольку генетические методы позволяют идентифицировать циркулирующие штаммы по их уникальным характеристикам. Положительный эффект применения методов генотипирования с высокой

разрешающей способностью был продемонстрирован на различных коллекциях изолятов возбудителя. Однако генетическое разнообразие может изменяться со временем, и данные о временной динамике генетического разнообразия представляют интерес для изучения эволюции патогена. В данном исследовании мы провели генотипирование MLVA16 штаммов *B. melitensis*, выделенных в 2016 и 2019 годах в Жамбылской области Казахстана. Полученные результаты свидетельствуют о значительной популяционной динамике возбудителя, так, как только 19% штаммов 2016 г. имели гомопластические аналоги среди изолятов штаммов 2019 г. Для получения достоверной картины динамики популяции и изменений в генетическом разнообразии штаммов бруцелл необходимо увеличение размера выборки и времени наблюдения.

Ключевые слова: *Brucella melitensis*; генотипирование; MLVA; Казахстан; кластерный анализ.

2016 ЖӘНЕ 2019 ЖЫЛДАРЫ ЖАМБЫЛ ОБЛЫСЫНДА БӨЛІНГЕН *B. MELITENSIS* ШТАМДАРЫНЫҢ ГЕНЕТИКАЛЫҚ ӘРТҮРЛІЛІГІ

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Түйін

Бруцеллез – адамға әсер ететін қауіпті зооноздық инфекция. Бруцеллез ауруының қоздырғышы бүкіл әлемде кең таралған және мал шаруашылығы мен халықтың денсаулығына зиянын тигізеді. Генетикалық типтеу әдістері бруцеллез қоздырғышының таралуын эпидемиологиялық қадағалаудың ажырамас құрамдас бөлігі болды, өйткені генетикалық әдістер айналымдағы штаммдарды бірегей сипаттамалары бойынша анықтауға мүмкіндік береді. Жоғары ажыратымдылықтағы генотиптеу әдістерінің оң әсері патогендік изоляттардың әртүрлі коллекцияларында көрсетілді. Дегенмен, генетикалық әртүрлілік уақыт өте келе өзгеруі мүмкін, ал генетикалық әртүрліліктің уақытша динамикасы туралы деректер патогендік эволюцияны зерттеу үшін қызығушылық тудырады. Бұл зерттеуде біз Қазақстанның Жамбыл облысында 2016 және 2019 жылдары бөлінген *B. melitensis* штаммдарының MLVA16 генотипін жасадық. Алынған нәтижелер қоздырғыштың айтарлықтай популяциялық динамикасын көрсетеді, өйткені 2016 жылғы штаммдардың 19% ғана 2019 жылғы штаммдардың изоляттары арасында гомопластикалық аналогтарға ие болды. Популяция динамикасының және *Brucella* штаммдарының генетикалық әртүрлілігіндегі өзгерістердің сенімді мәліметін алу үшін, сынама көлемін және бақылау уақытын ұлғайту қажет.

Кілт сөздер: *Brucella melitensis*; генотиптеу; MLVA; Қазақстан; кластерлі талдау.