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RESEARCH

Study of epidemiological and molecular characteristics of *Brucella* strains circulating in Kazakhstan

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Abstract
Brucellosis is one of the most common zoonotic infectious diseases of animals. The causative agent of brucellosis is a highly conserved bacterium of the genus *Brucella*, which includes 14 species with minor genetic changes. Our work aimed to study the epidemiological and molecular characteristics of *Brucella* strains circulating in Kazakhstan, including the study of polymorphisms in critical virulence genes using single nucleotide polymorphism (SNP) analysis based on whole genome sequencing (WGS). A total of 21 *Brucella* isolates obtained in 2023–2024 from cattle, sheep and goats, horses, camels, pigs, dogs, and humans were analyzed. As a result of whole-genome sequencing, 69 virulence genes were detected. The genome of *Brucella melitensis*, the most virulent species of *Brucella*, was the most susceptible to changes. Among the 10 virulence genes studied using SNP, polymorphisms were found in 7 genes. Some genetic mutations resulted in amino acid changes in the coding sequences of these virulence genes. Knowledge of virulence factors and changes in their coding genes has practical implications for epidemiological studies in outbreak areas, their management, and the implementation of brucellosis control strategies.

Keywords Virulence factors · Virulence genes · Brucellosis · Single nucleotide polymorphism · Whole genome sequencing

Introduction

Brucellosis is one of the most common zoonotic infectious diseases (Edao et al. 2023), especially in regions with intensively developed livestock farming, such as the Republic of Kazakhstan.

Bacteria of the genus *Brucella* cause brucellosis. This disease significantly reduces livestock productivity, thereby affecting the country's economy. According to the Food and Agriculture Organization of the United Nations (FAO), brucellosis is currently recognized as a significant public health problem that poses a real threat to the biological security of the population in the regions of the Mediterranean, South, and Central America, Africa, Asia, the South Caucasus, the Arabian Peninsula, India, and the Middle East (FAO 2006). According to the latest data, 1.6–2.1 million new human brucellosis cases are registered worldwide yearly (Laine et al. 2023). The annual incidence rates of brucellosis per 1,000,000 populations in several countries endemic for brucellosis are, on average, Saudi Arabia −214.4; Iran −238.6; Turkey −262.2; Iraq −278.4; Syria −1603.4. According to the World Health Organization (WHO), in regions unfavorable to brucellosis, the incidence rate can vary from 10 to 25 times higher than that of the officially registered one (WHO 2020). In Russia, the incidence rates of brucellosis have consistently been 0.2–0.7 per 100,000 population in recent decades. In Eastern European countries, the number of

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disease cases in people ranges from 21 to 64 per 1,000,000 people. In some endemic countries, the prevalence of human brucellosis exceeds 10 per 100,000 populations (Yang et al. 2020). Brucellosis is endemic in Africa, Asia, and Europe; for example, *B. melitensis* is the predominant species in Egypt since sheep are the most common ruminant species (El-Diasty et al. 2021). In Iraq, *B. abortus* and *B. melitensis* were the dominant pathogens (Ilyas et al. 2024). *B. melitensis* is the most common *Brucella* species in the Mediterranean Basin and Turkey. Among the countries in southern Europe, Greece has the highest incidence of human brucellosis (Brangsch et al. 2023).

The Central Asia and Eastern Europe countries of the Commonwealth of Independent States (CIS) have some of the highest human and animal brucellosis rates globally. Central Asia, including Kazakhstan, Kyrgyzstan, and Tajikistan, has the highest incidence of human brucellosis worldwide (Liu et al. 2024). In the neighboring country of Kyrgyzstan, 89 human isolates were studied and assigned to the *B. melitensis* species of the eastern Mediterranean group (Kydyshov et al. 2022a, b). This is consistent with our previous study, in which the *B. melitensis* circulating in the Republic of Kazakhstan also belongs to the eastern Mediterranean group (Daugaliyeva et al. 2018).

Most frequently, new outbreaks of human brucellosis are observed in Central Asia, including the Republic of Kazakhstan (Aikimbaev et al. 2021; Kydyshov et al. 2022a, b; Karakululy et al. 2023). Taipova et al. (2023) claim that Kazakhstan is among the 25 countries with the highest incidence rates of brucellosis (Taipova et al. 2023). According to Urazaeva et al. (Urazaeva et al. 2018), a high incidence rate of brucellosis in the population is also noted in the countries neighboring Kazakhstan - Iraq, Tajikistan, Saudi Arabia, Iran, and Kyrgyzstan.

According to Syrym et al. (Syrym et al. 2019), in Kazakhstan, there is an increase in the incidence of bovine brucellosis in West Kazakhstan, Karaganda, Kostanay, and Pavlodar regions. There is an increase in small ruminants in the Zhambyl, East Kazakhstan, and Almaty regions. The lowest incidence rate is in the Mangistau region. Yespembetov et al. (Yespembetov et al. 2019) indicate that the causative agent of bovine brucellosis *B. abortus* was isolated from the northern regions of Kazakhstan (more than 90% of the total number of samples), and the causative agent of sheep and goats' brucellosis *B. melitensis* was registered in the southeast.

The above data make research on the study of the brucellosis agent's infection sources and virulence factors relevant and priority areas for medicine and veterinary science in Kazakhstan.

Based on biochemical characteristics and host preferences, 12 genetically close *Brucella* species have been identified. *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B. suis* (pigs, reindeer, and hares), *B. ovis* (rams), *B. neotomae* (desert rats), *B. canis* (dog), *B. ceti* (cetaceans), *B. pinnipedialis* (pinnipeds), *B. microti* (voles), *B. papionis* (baboons), *B. vulpis* (foxes), *B. amazoniensis* (humans) (Daugaliyeva et al. 2024; Liu et al. 2025). Two more *Brucella* species have also been added to the *Brucella* classification. Thus, a Gram-negative bacterium (strain BO1(T) was isolated from a breast implant infection of a 71-year-old female patient with clinical signs of brucellosis. Due to the unexpected isolation of the bacterium, it was proposed to name it *B. inopinata* (Scholz et al. 2010). Another new pathogenic species, *B. nosferati* sp. nov., was isolated from bat tissues, including salivary glands (Hernández-Mora et al. 2023). The genus *Brucella* includes highly conserved bacteria with slight genetic variation. However, cross-infection of secondary hosts with *Brucella* has been reported. Moreover, cross-infection of animals has been observed on farms where cattle and small ruminants are housed together. Most human infections are caused by *B. melitensis*, *B. abortus*, *B. suis*, and occasionally *B. canis*. The most severe human diseases are caused by *B. melitensis* and *B. suis* (except biovar 2). *B. melitensis* is the most common and virulent species, sometimes causing death. The *Brucella* genome is conservative and consists of 2 circular chromosomes of 1.2 and 2.1 Mbp, except *B. suis* biovar 3, which has 1 chromosome of 3.1 Mbp. In addition, most essential genes are located in Chr I (Halling et al. 2005).

All the advances in genome sequencing (WGS) technology allow for pan-genome assessment of *Brucella* genome variability, including virulence genes. This allows for rapid comparison with virulence factor databases (VFDB, http://www.mgc.ac.cn/VFs/main.htm) and investigation of how these genes differ among strains (Rabinowitz et al. 2021). Many virulence genes can be present in different strains, so WGS should be accompanied by core genome single nucleotide polymorphism (cgSNP, WGS–SNP) analysis.

Our work aimed at studying the epidemiological and molecular characteristics of *Brucella* strains circulating in Kazakhstan, including polymorphisms in critical virulence genes. This knowledge is necessary for epidemiological studies in outbreak areas, their management, and implementing brucellosis control strategies.

Materials and methods

Isolates of *Brucella* spp. detected in Kazakhstan

In the period 2023–2024, serological monitoring of animals (cattle and sheep and goats, camels, horses, pigs, dogs) was carried out on the territory of the Republic of Kazakhstan

(in the regions unfavorable for brucellosis: northern, southern, western, and central), as a result of which 155 positive results were obtained out of 2981 tested samples using the Rose Bengal test (RBT) and the complement fixation test (CFT). Positively reacting animals were sent for slaughter, from which samples of pathological material were taken for research (blood, parenchymatous organs, and aborted fetuses). Bacteriological studies were carried out in the Brucellosis Laboratory of the Veterinary Institute. During bacteriological studies, selectively modified Farrell's medium was used to isolate *Brucella* cultures, which were prepared by adding to 1 L of agar: polymyxin B sulfate (5000 units = 5 mg); bacitracin (25,000 units = 25 mg); natamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 units); vancomycin (20 mg). The necessary tests were carried out according to the recommendations of the World Organization for Animal Health (WOAH) (Terrestrial Animal Manua, 2015; Terrestrial Animal Manual 2022): assessment of the morphology of the microorganism after Gram staining, observation of the morphology colonies by the White - Wilson method, evaluation of growth characteristics, urease and oxidase tests, plate agglutination reaction with polyclonal ant brucellosis serum. Biovars and species were identified using Tbilisi (Tb) phage lysis and agglutination with monospecific sera against A, M, or R antigens. The need for atmospheric carbon dioxide enrichment for bacterial growth, H_2S production (determined by lead acetate paper), and growth in the presence of basic fuchsin and thionine were also determined. Twenty-one pure cultures of *Brucella* spp. were isolated in this manner. To deprive the isolates of infectious properties, the laboratory staff subjected the cultures to thermal destruction at 80 °C for 10 min. Genomic DNA from *Brucella* spp. was isolated in the Laboratory of Molecular Genetics of Microorganisms, Institute of Microbiology and Virology, using the PureLink® Genomic DNA Kits (Invitrogen, USA) according to the manufacturer's protocol for gram-negative bacteria. DNA concentration was determined using a Qubit fluorimeter (Invitrogen, USA) on a scale for dsDNA HS using the Qubit® dsDNA HS Assay Kit (Invitrogen, USA). Whole-genome sequencing was then performed using the Illumina MiSeq platform. Briefly, genetic libraries for whole genome sequencing were prepared as follows: 1 ng of DNA was labeled with Nextera XT transposase, amplified libraries were purified and quality checked on an Agilent Bioanalyzer 2.0 (Agilent Technology, Hamburg, Germany) using the High Sensitivity DNA Reagents DNA analysis kit (Agilent Technology, Lithuania) (Gautam et al. 2019). DNA libraries diluted to 4 nM concentrations were sequenced using the V3 reagent kit (600 cycles) on the Illumina MiSeq platform.

Bioinformatics analyses

Next-generation sequencing (NGS) runs were downloaded from the Illumina BaseSpace cloud server. The CLC Genomics Workbench (GW) v. 24 bioinformatics pipeline was used to analyze the whole genome sequencing (WGS) data. The pipeline input consisted of Illumina FASTQ paired-end sequencing files and metadata. Specifically, CLC Genomics Workbench v. 24 software was used to assess the quality of the sequenced reads: adapters and artificial bases remaining after the sequencing reaction were trimmed. The quality filtering tool cut the 5' and 3' ends of each read if there were more than two ambiguous bases (i.e., N) in a row, applying the read > Q28 criterion, or when the base accuracy (i.e., based on the sum of phred quality scores) was below 95%. The sequence filter removed reads shorter than 15 bases in length 15.

The paired-end trimmed reads of *Brucella* spp. strains were then assembled de-novo. Kmer sizes 21, 33, 55, 77, 99, and 127 were used for assembly, with a minimum contig length of 500 bp. Potential contaminants in each assembled genome were also assessed. The Create Whole Genome Alignment tool in CLC Genomics Workbench v. 24 aligned the genomes (Chiaromonte et al. 2002). To avoid false matches to repetitive regions in the genome, we combined the HOXD substitution score with a Kmer frequency-based correction term, similar to Progressive Mauve. The resulting *Brucella* genomes were submitted to the NCBI Genome Repository for the BioProject NCBI PRJNA1223166.

The Create Average Nucleotide Identity Comparison tool in CLC Genomics Workbench v. 24 was used to obtain a quantitative measure of similarity between genomes. A whole genome alignment was taken as input, and regions aligned between the two genomes were identified for each pair of genomes. The following two measures were calculated: average nucleotide identity as the percentage of exactly matching nucleotides for the genomes and a distance matrix showing the number of nucleotide differences between the genomes.

Single nucleotide polymorphism (SNP) typing was performed using CLC Genomics Workbench v. 24, which calculated the number of nucleotide differences between the genomes included in the analysis (Kaas et al. 2014). However, SNP-based typing requires a defined reference and is more difficult to compare between different assays and for more distantly related samples. CLC Genomics Workbench performs a core genome SNP analysis, meaning that only SNPs found in regions matching all samples included in the SNP-based typing analysis are considered. The differences found are then used to construct a phylogenetic tree using the unweighted pair group method with arithmetic mean (UPGMA) and the Neighbor-Joining method. For

comparative analysis, reference genomes were selected based on their high-quality assembly, completeness of annotation, and representation of known genetic diversity within the genus *Brucella*: GCF_000369945.1 *Brucella abortus* Bruc_abor_544_V1, Scaffold; GCF_000007125.1 *Brucella melitensis* ASM712v1, Complete Genome; GCF_000007505.1 *Brucella suis* ASM750v1, Complete Genome; GCF_000016845.1 *Brucella ovis* ASM1684v1, Complete Genome; GCF_000018525.1 *Brucella canis* ASM1852v1, Complete Genome.

CLC Genomics Workbench v. 24 software was used to identify mutations in *Brucella* isolates. Subsequently, high-quality reads were aligned using the Read Alignment to Reference tool and the standard CLC alignment algorithm. To address chemistry-related issues and variations in read lengths, default settings were implemented during alignment for match and mismatch scores and insertion and deletion costs. To be included in the final alignment reads had to match the reference at > 50% of sites; of these sites, 80% had to be identical.

The heatmap was generated using the "Generate Heatmap from Alignment" tool implemented in CLC Genomics Workbench v. 24. The analysis creates a heat map from pairwise comparisons, such as the average nucleotide identity matrix.

Potential virulence-associated genes were identified by querying the virulence factor database "VFDB, http://www.mgc.ac.cn/VFs/ (accessed July 30, 2024) " (Liu et al. 2019).

Detection of genetic mutations localized in 10 virulence factor (VF) genes (*Ure*, *Omp19*, *mviN*, *VceC*, *prpA*, *betB*, *bpe275*, *bspB*, *perA*, *manA*) was performed using different bioinformatics tools. *Brucella* genome consensus sequences were exported from CLC Genomics Workbench into FASTA files and then examined using BioEdit software v. 7.2.5. SNP typing results were combined with annotation information from reference sequences and NCBI resources. VF sequences were isolated, and genetic variations were tested for amino acid changes in the coding sequences.

Identification of significant virulence genes using PCR

PCR was performed in a Mastercycler Nexus Gradient thermal cycler (Eppendorf, Germany). The reaction mixture (25 µl) for the *Virb* and *Ure* gene contained 12.5 µl DreamTaq Hot Start PCR (2X) Master Mix (Thermo Fisher, USA), 1.5 µl of each primer (10 pM), 50 ng DNA, and water up to 25 µl (Mirnejad et al. 2017; Papaparaskevas et al. 2023).

The amplification mode for *Virb* was 95 °C for 4 min, followed by 35 cycles consisting of 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1.3 min. The final elongation was performed at 72 °C for 10 min.

The amplification regime for *Ure* was 95 °C for 4 min, followed by 35 cycles of 95 °C for 1 min, 65 °C for 1 min, and 72 °C for 1.3 min. The final elongation was at 72 °C for 10 min.

The amplified product was separated in a 1% agarose gel; bands were stained with ethidium bromide and visualized on an Infinity VX2 gel documentation system (Vilber Lourmat, Belgium). 1xTAE buffer was used as electrode buffer.

Multiplex PCR for the *Omp 19*, *MviN*, *ManA*, *PerA*, and *WbkA* genes was performed in a volume of 25 µl: 12.5 µl DreamTaq Hot Start PCR (2X) Master Mix (Thermo Fisher, USA), 0.5 µl of each primer (10 pM), 50 ng DNA, and water up to 25 µl. Amplification mode: at 95 °C for 5 min, then 35 cycles consisting of 95 °C for 1 min, 58.5 °C for 45 sec., 72 °C for 1 min. The final elongation was performed at 72 °C for 8 min. The amplified product was separated in a 1.2% agarose gel.

The reference strains *B. abortus* 544 and *B. melitensis* 16 M were used as positive controls, and Actinomyces spp. was used as a negative control.

Results

Epidemiological situation of brucellosis in Kazakhstan

The Republic of Kazakhstan is endemic for animal and human brucellosis. Animal brucellosis is registered annually in Kazakhstan. Cattle and small ruminants are most susceptible to this disease (Table 1). As of August 1, 2024, the cattle population amounted to 966.5 thousand heads, sheep − 1,327.4 thousand heads, and goats − 196.8 thousand heads.

The epidemiological situation for brucellosis in Kazakhstan was studied based on the analysis of official veterinary reporting data of the Republican Veterinary Laboratory of the Committee for Veterinary Control and Supervision of the Ministry of Agriculture of the Republic of Kazakhstan and the Scientific and Practical Center for Sanitary and Epidemiological Expertise and Monitoring of the National Center for Public Health of the Ministry of Health of the

Table 1 Brucellosis incidence rates of cattle and small ruminants in the Republic of Kazakhstan for 2019–2023 (in absolute and relative values)

Animal species	2019		2020		2021		2022		2023	
	Number of cases	%	Number of cases	%	Number of cases	%	Number of cases	%	Number of cases	%
Cattle	36,899	0,4	33,933	0,32	30 297	0,28	21 898	0,18	17 016	0,16
Small ruminants	13,255	0,07	9633	0,04	7 417	0,03	5 197	0,02	4 875	0,01

Republic of Kazakhstan. The results of the analysis are presented in Tables 1 and 2.

As Table 1 shows, the incidence of farm animals is gradually decreasing. However, it should be noted that out of 17 regions of Kazakhstan, the incidence of cattle and small cattle is observed in 16 regions, except the Mangistau region, which confirms the spread of the disease throughout the Republic. The absolute number of people infected with brucellosis also gradually decreased from 2019 to 2023 (Table 2).

It should be noted that despite the decrease in the absolute number of people infected with brucellosis, the relative incidence rate per 100 thousand population remained high and ranged from 4.61 in 2019 to 2.31 in 2023. Population incidence is observed everywhere in all 17 regions of the republic, including the Mangistau region, where animal brucellosis has not been registered. The above indicators are alarming for medical and veterinary specialists, who urgently need to study the epidemiological process of this infection to develop and organize optimal scientifically based anti-brucellosis measures.

Isolation of pure brucellosis cultures

Viewing smears stained by Gram showed that the cells morphology is highly stable. The studied microorganisms were gram-negative, red, pink, small, short rods with rounded ends, ovoids, and coccobacilli, which were located singly, less often in pairs or small groups. They were immobile and unstable to acids. They did not form specialized cells (spores and capsules).

To determine the presence of dissociated cells in the culture, the cultures were seeded on the agar surface in Petri dishes. After 4 days, the culture material was stained using the White - Wilson method with crystal violet at a dilution of 1:2000. Smooth colonies were stained light yellow with a bluish rim along the edge, which also confirmed the absence of dissociation of the studied brucellae and their presence in the S-form.

Brucella species identification using whole genome sequencing (WGS)

The assembled genomes were evaluated using BLAST. The first bioinformatics step yielded some interesting findings. Some isolates were incorrectly classified by classical bacteriology, being assigned to the wrong species (in one case, also to the wrong genus). Based on this, an updated metadata table was required to continue further analyses. The final panel of samples is presented in Table 3.

As shown in Table 3, mismatches were found in 6 species, and a mismatch of the genus was detected.

The CLC Genomics Workbench v. 24 sample report shows that all samples were of good quality with a sequence depth from 47x to 186x, which was satisfactory for further mutation analysis (Supplementary Table 1).

The results of genome alignment showed that the studied *Brucella* isolates do not contain internal rearrangements, i.e., do not carry large-scale events such as inversions and translocations.

Creation of evolutionary trees and heat maps. Identification of SNPs based on comparison with the reference genome

SNPs identified by WGS were used to generate a phylogenetic tree using the unweighted pair group method with arithmetic mean (UPGMA) and the Neighbor-Joining method (Figs. 1 and 2).

Figure 1 shows the UPGMA phylogenetic tree, including all *Brucella* isolates and metadata. This tree clearly shows the separate clustering of *Brucella* species, with *B. ovis* being the most divergent. The tree shows the average nucleotide distances between samples and sample clusters as integers.

The cluster of *B. abortus* isolates includes the reference strain Ba544 and three closely related isolates from cattle ($n=2$) and camel ($n=1$). One human isolate (Bm487) is the most distant, branching off from its branch at the root of the *B. abortus* tree.

Interestingly, two *B. melitensis* isolates from the same village (Nurlykent) but from different hosts (sheep and dog) were closely related, thus worthy of further epidemiological studies.

The genetic distance between the two *B. suis* field isolates and the reference strain is minimal, as indicated by the average nucleotide distance shown in the tree.

The Neighbor-Joining phylogenetic tree included all *Brucella* isolates. The radial visualization of the tree, based on the whole genome alignment distance matrix, clearly shows the separation of *Brucella* species. Furthermore, the

Table 2 Brucellosis incidence rates among the population of the Republic of Kazakhstan for 2019–2023

2019		2020		2021		2022		2023	
Number of cases	Indicators per 100 thousand	Number of cases	Indicators per 100 thousand	Number of cases	Indicators per 100 thousand	Number of cases	Indicators per 100 thousand	Number of cases	Indicators per 100 thousand
842	4,61	504	2,76	448	2,36	164	2,20	171	2,31

Table 3 *Brucella* species identified by bacteriological and molecular genetic studies

ID	Animal species	Species by bacteriology	Species by WGS
Ba 544	control	*Brucella abortus*	*Brucella abortus*
Ba4556	camel	*Brucella abortus*	*Brucella abortus*
Ba48	cattle	*Brucella abortus*	*Brucella abortus*
Ba732	cattle	*Brucella abortus*	*Brucella abortus*
Ba44	cattle	*Brucella abortus*	*Brucella abortus*
Ba134	cattle	*Brucella abortus*	*Brucella abortus*
Bm46	pig	*Brucella abortus*	*Brucella abortus*
Ba93	small ruminant	*Brucella abortus*	*Brucella melitensis*
Bm 16	control	*Brucella melitensis*	*Brucella melitensis*
Bm499	small ruminant	*Brucella melitensis*	*Brucella melitensis*
Bm487	human	*Brucella melitensis*	*Brucella abortus*
BmM17	small ruminant	*Brucella melitensis*	*Brucella abortus*
Bm909	small ruminant	*Brucella melitensis*	*Brucella suis*
Bm90Dg	dog	*Brucella melitensis*	*Brucella melitensis*
Bm68	horse	*Brucella melitensis*	*Brucella melitensis*
Bm3101	small ruminant	*Brucella melitensis*	*Brucella melitensis*
Bm41	small ruminant	*Brucella melitensis*	*Brucella melitensis*
BmB1	cattle	*Brucella melitensis*	*Brucella abortus*
BT2024	small ruminant	*Brucella melitensis*	*Brucella melitensis*
Bm4	small ruminant	*Brucella melitensis*	*Brucella melitensis*
Bo63-290	control	*Brucella ovis*	*Bordetella bronchiseptica*
Bo873	small ruminant	*Brucella ovis*	*Brucella ovis*
Bs1330	control	*Brucella suis*	*Brucella suis*
Bs4	pig	*Brucella suis*	*Brucella suis*
Bc1066	dog	*Brucella canis*	*Brucella canis*

Neighbor-Joining method confirms that *B. ovis* is the most divergent species.

Finally, the phylogenetic topology of the studied isolates was confirmed by the maximum likelihood (ML) analysis, as the resulting cladogram based on the significant SNPs again showed the clustering of *Brucella* isolates according to species; in particular, the ML phylogeny highlighted the divergence of the human *B. abortus* isolate Bm487 from the central *B. abortus* cluster (Fig. 3).

Figure 4 shows a heat map based on the average nucleotide identity matrix showing Brucella isolates' similarity and phylogenetic relationships.

Figure 4 shows that the *Brucella* species form distinct clusters. The similarity threshold was 95% based on the highly conserved *Brucella* genome. *B. ovis* and *B. suis* appear more diverse than the *B. abortus*, *B. melitensis*, and *B. canis* clusters.

The highest number of mutations were found in the *B. melitensis* genome, as shown in Table 4, which was constructed from a VCF (Variant Calling Format) file. After alignment, the Export Mapping Coverage tool was used to export the coverage of each nucleotide (A, T, C, G, or N) for each SNP, with the SNP locations listed in the resulting VCF files. The VCF files show a list with each mutation, compared to a reference, for each *Brucella* chromosome. Since the reference is the same for each *Brucella* species, the numbering is the same, meaning that the SNP results are comparable between samples within a species.

However, it should be noted that the data on *B. suis*, *B. ovis*, and *B. canis* were insufficient for a thorough comparison with *B. melitensis* and *B. abortus*. Further studies in this direction may be needed.

Identification of virulence genes

Most of the identified genes were in the group of virulence factors associated with the lipopolysaccharide (LPS) operon. Another significant pool of virulence factor genes was represented by genes encoding the type IV secretion system (*Vir*B1-B12). Genes (*BtpA*, *BtpB*) encoded proteins containing the toll-interleukin-1 receptor (TIR) domain, which inhibited dendritic cell maturation and anti-inflammatory cytokine production, enhancing immune evasion, the *ricA* gene encoded the Rab2-interacting conserved protein A, and the *cgs* gene was associated with the production of cyclic β−1,2-glucans. These results are consistent with those recently published by Dadar et al. (Dadar et al. 2023) on *Brucella* isolates from Iran.

Supplementary Tables 2 and Table 5 provide an overview of the 69 virulence factor (VFs) genes found in the analyzed *Brucella* isolates.

Detection of significant virulence genes by PCR

PCR and multiplex PCR methods were used using specific primers for *VirB*, *Ure*, *WbkA*, *Omp19*, *MviN*, *ManA*, and *PerA* genes, amplified in sizes of 881, 1282, 931, 550, 344, 271, and 716 bp, respectively (Mirnejad et al. 2017; Papaparaskevas et al. 2023). The distribution of virulence-specific

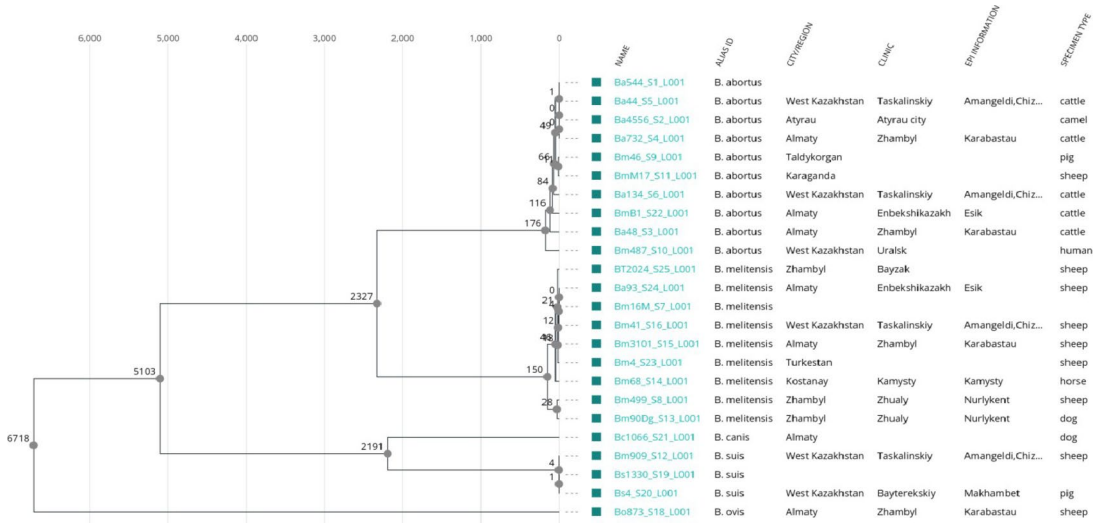

Fig. 1 Phylogenetic tree using UPGMA method

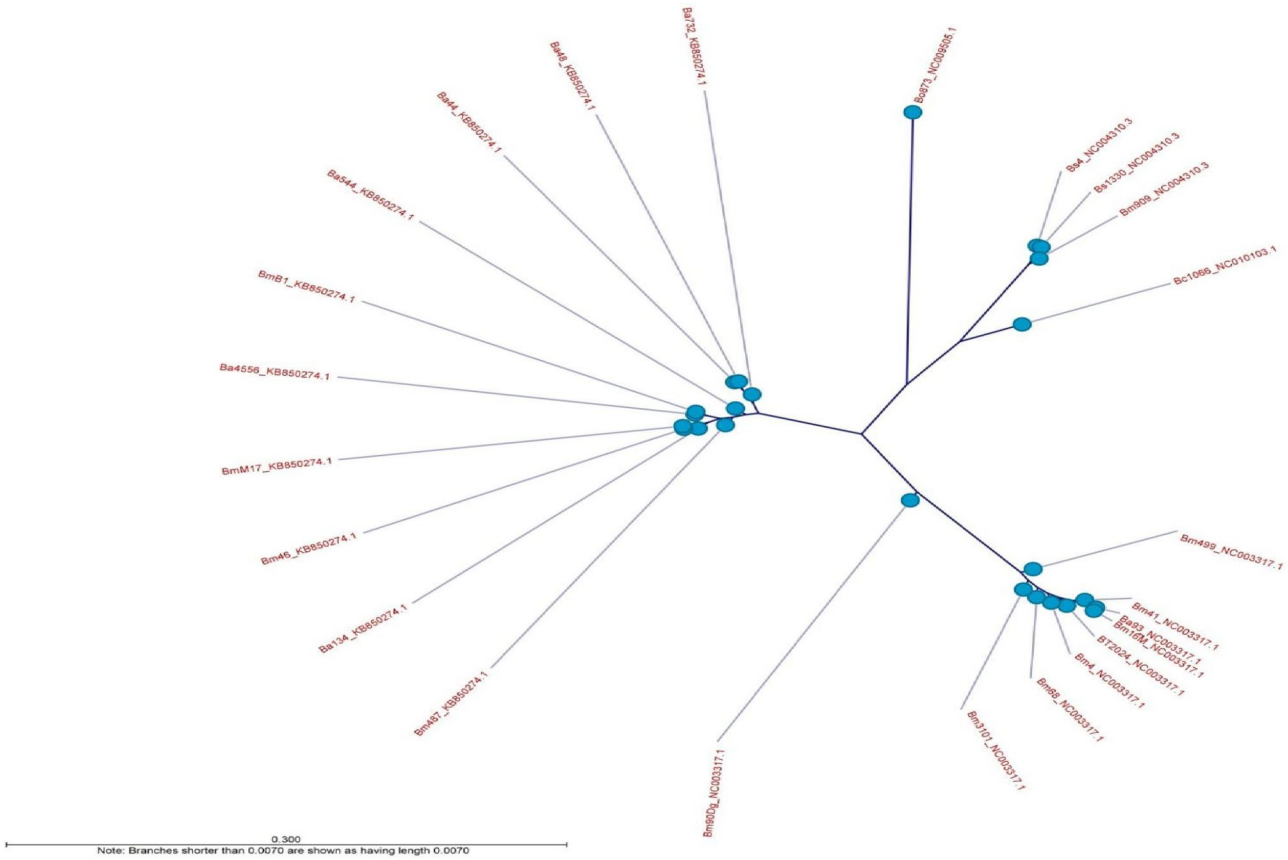

Fig. 2 Phylogenetic tree using the Neighbor-joining method

genes in the studied animal samples showed that all strains (100.0%) carried *VirB*, *Ure*, *WbkA*, *Omp19*, *MviN*, *ManA*, and *PerA* genes, respectively. The virulence genes and their associated bands obtained by PCR and multiplex PCR are presented in Figs. 5, 6, 7, 8, and 9.

Mutation analysis of significant virulence factor genes (*VceC*, *BPE275*, *BspB*, *Omp19*, *Ure*, *MviN*, *PrpA*, *BetB*, *PerA*, *ManA*)

Analysis of SNP variations in 10 virulence genes showed that 3 out of 10 evaluated genes (*BPE275*, *BspB*, *Omp19*)

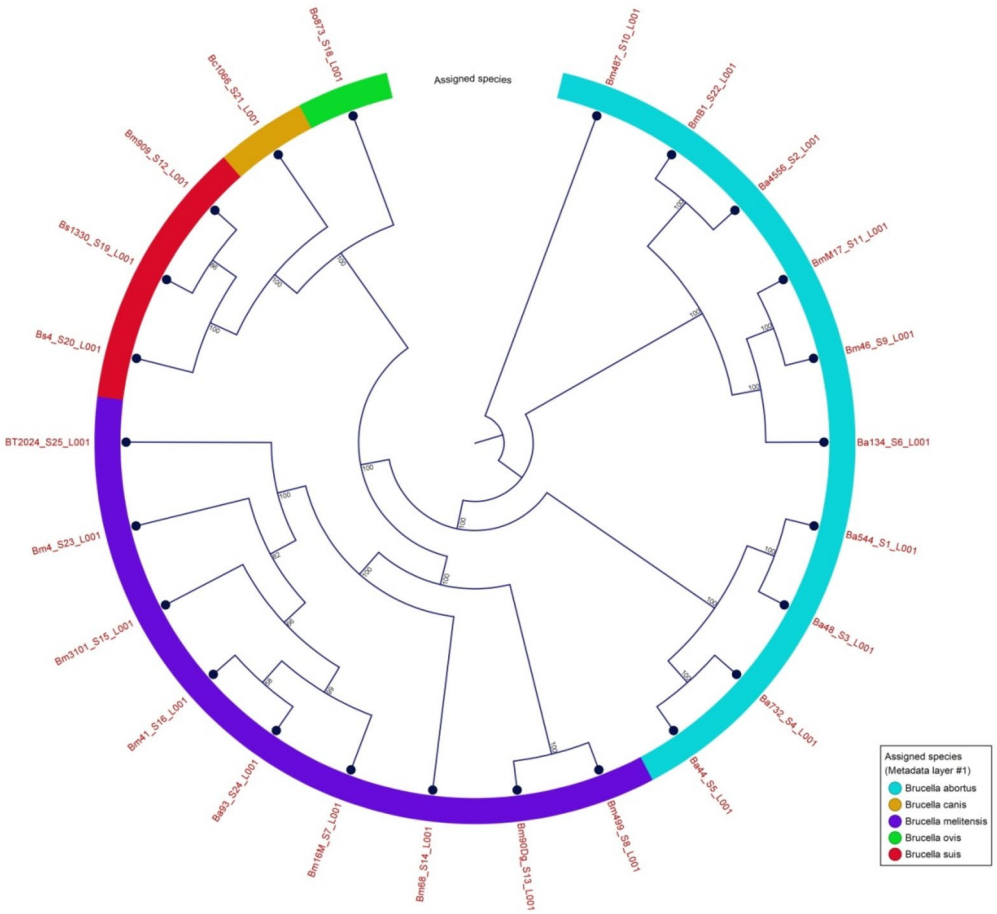

Fig. 3 Circular cladogram using maximum likelihood. Bootstrap analysis (1000 replicates) was performed to test the robustness of branches, and the resulting values are shown in the internal nodes

were conserved entirely, without any mutations. The results of mutation analysis by *Brucella* species are presented in Table 6.

The *perA* gene had one mutation (position 1.062.358), and the *VceC* gene had one mutation (position 118.418). Mutations in the *ManA* gene were synonymous. Mutation was observed in the *BetB* gene in 8 *B. melitensis* strains, the *PrpA* and *MviN* genes in 9 *B. melitensis* strains, and *Ure* gene in 6 *B. abortus* strains. Genetic mutations resulted in changes in amino acids in the coding sequences of virulence genes.

Discussion

Kazakhstan has been an endemic country for brucellosis of farm animals for a long time, ever since the collapse of the USSR. This is due to the country's intensive development of animal husbandry and non-compliance with sanitary regulations. However, the incidence of farm animals gradually decreased in cattle from 0.4% in 2019 to 0.16% in 2023 and sheep and goats from 0.07% in 2019 to 0.01% in 2023. As can be seen from the above data, the disease is more common in cattle than in sheep and goats. As a result of the spread of the disease, there is a constant risk of infection in the country's population. Despite the decrease in the absolute number of people infected with brucellosis, the relative incidence rate per 100 thousand population remained high and ranged from 4.61 in 2019 to 2.31 in 2023.

Over two years, we isolated 21 cultures from various animal species (cattle, sheep and goats, horses, camels, pigs, dogs, and humans) from different country regions. As a result of the conducted studies, nine samples of the species *B. abortus*, eight samples of the species *B. melitensis*, two samples of the species *B. suis*, and one sample each of the species *B. canis* and *B. ovis* were identified. The species *B. abortus*, in addition to the primary host of cattle, was identified in camels, pigs, humans, and sheep and goats. The species *B. melitensis*, in addition to the primary host of sheep and goats, was identified in dogs and horses. The species *B. suis*, in addition to the primary host of pigs, was identified in sheep and goats. It was also found that the strain of the

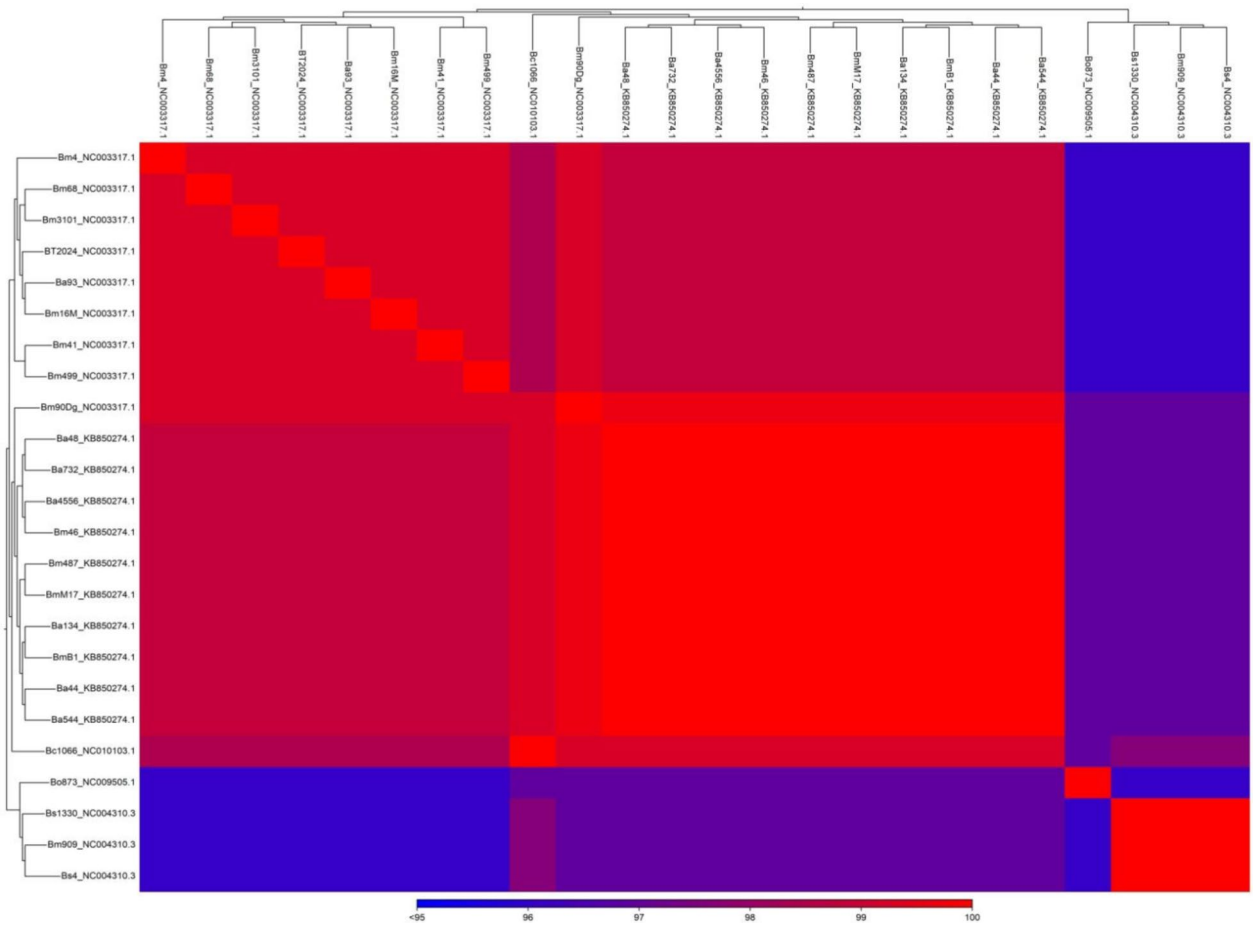

Fig. 4 Heat map isolate *Brucella*

species *B. ovis* differed from other species. Also, the strain of *B. abortus* isolated from humans differed from different strains of the species *B. abortus*.

Studying multiple genes and polymorphisms in critical genes is necessary to understand *Brucella* virulence's variability better.

Brucella virulence factors are located on the pathogen's cell membrane and allow survival inside macrophages. The main virulence factor of *Brucella* is lipopolysaccharide (LPS), located on the cell surface of the pathogen. The smooth phenotype (S-form) is formed by complete LPS, consisting of lipid A, oligosaccharide, and O-side chains of the polysaccharide. LPS of rough strains (R-form) does not contain O-side chains (Pappas et al. 2006). Antigenic determinants (O-chains of polysaccharides) are considered factors influencing the virulence of *Brucella*, necessary for invasion and survival in host cells. Virulence factor genes are responsible for host immune evasion, involvement in LPS synthesis, maturation and function, intracellular survival, regulation and expression of the type IV secretion system (T4SS) (Karthik et al. 2021), regulation of the BvrR/BvrS system and outer membrane proteins (Yang et al. 2020).

The expression of several cell wall molecules, such as integral membrane-bound protein (MviN), mannose-6-phosphate isomerase (ManA), mannosyltransferase (WbkA), perosamine synthetase (PerA) and outer membrane protein 19 (Omp19), contributes to the control of intracellular pathogen transport and the initial survival of bacteria in macrophages and other cells of the reticuloendothelial system (Papaparaskevas et al. 2023).

The *Ure* gene is responsible for the production of the nickel-containing enzyme urease. Urease catalyzes the breakdown of urea into ammonia, which is oxidized by oxygen to produce nitrogen, which is essential for bacteria. Ammonia allows pathogens to survive in the stomach's acidic environment in vivo and in vitro (Papaparaskevas et al. 2023).

In addition, *Brucella* produces cyclic β (1–2)-glucan (encoded by the *Cbg* gene), a low-molecular-weight polysaccharide also known as B-polysaccharide, which interacts with lipids and promotes pathogen survival by preventing phagosome-lysosome fusion (Papaparaskevas et al. 2023).

Table 4 Genetic mutation compared to *Brucella* reference strains

Isolate ID	Deletion	Complex	Single Nucleotide		Multiple Nucleotide	
Insertion			Total		Polymorphisms	
			Polymorphism			
Genetic variations versus Brucella abortus 544 (GCF_000369945.1)						
Ba134	1267	5	61	73	13	1419
Ba544	73	0	5	8	3	89
BmB1	1242	6	63	67	12	1390
Bm46	1258	6	57	68	11	1400
BmM17	1257	7	62	69	10	1405
Ba44	435	0	28	25	8	496
Ba732	185	1	16	10	2	214
Ba4556	1235	7	63	70	10	1385
Ba48	98	1	8	15	4	126
Bm487	1236	6	64	64	11	1381
Genetic variations versus *Brucella melitensis* 16 M (GCF_000007125.1)						
BT2024	2467	31	123	167	64	2852
Bm41	2472	39	123	169	56	2859
Bm3101	2441	30	117	158	60	2806
Bm68	2459	38	126	167	56	2846
Bm499	2444	35	128	158	60	2825
Bm16M	2465	37	121	167	58	2848
Ba93	2471	38	127	169	57	2862
Bm90Dg	1966	31	108	129	56	2290
Bm4	2475	35	123	165	59	2857
Genetic variations versus *Brucella suis* (GCF_000007505.1)						
Bs4	61	0	17	13	0	91
Bs1330	61	0	16	13	0	90
Bm909	64	0	16	14	0	94
Genetic variations versus *Brucella ovis* (GCF_000016845.1)						
Bo873	205	0	16	24	6	251
Genetic variations versus *Brucella canis* (GCF_000018525.1)						
Bc1066	40	0	1	7	0	48

T4SS, encoded by the *VirB* operon, regulates one of the most important virulence factors by secreting host cell effectors and regulators (Papaparaskevas et al. 2023). It plays a vital role in bacterial attachment, invasion, movement, survival, and replication in the host cell.

Many researchers have studied the virulence genes of *Brucella*.

For example, researchers from Iran found six virulence genes by PCR analysis in 57 *B. melitensis* and 21 *B. abortus* isolates with variable cycle: *Ure*, *WbkA*, *Omp19*, *MviN*, *ManA*, and *PerA* (74.4%, 89.7%, 93.6%, 94.9%, 100%, and 92.3%, respectively) (Mirnejad et al. 2017). A study of 60 *B. melitensis* isolates from Iran showed that the *BtpA* (also known as *TcpB*), *BtpB*, *VirB5*, *VceC*, *Bpe275*, *BspB*, and *VirB2* genes were present in 100% of the isolates, and *PrpA* and *BetB* were present in 86% and 97%, respectively (Rabinowitz et al. 2021). In our study, PCR of 21 *Brucella* isolates also confirmed the presence of the *VirB*, *Ure*, *WbkA*, *Omp19*, *MviN*, *ManA*, and *PerA* genes in 100% of all isolates.

Scientists from Egypt identified 43 virulence genes in *B. melitensis* and *B. abortus* strains, researchers from India also identified 43 virulence genes in *B. melitensis* strains (Khan et al. 2021; Karthik et al. 2021).

Scientists from China found 66 virulence genes in 2 strains (one from a marmot, the other from a human), except the *bmaA* and *btaE* genes (Xue et al. 2023).

In our study, using WGS, we identified 69 virulence genes in the studied *Brucella* strains. The genome of *B. melitensis*, the most virulent *Brucella* species, was the most susceptible to changes. In comparison with the work of Xue et al., we also did not identify the *bmaA* and *btaE* genes, except for the *B. suis* 909 strains, which had the *bmaA* virulence gene. In contrast to PCR, WGS showed that the *VirB*10 gene was absent in all *B. melitensis*, *B. suis*, and 2 *B. abortus* strains but was present in *B. ovis* and *B. canis* strains. *B. ovis* 873 strain lacks the *Pmm*, *WboA*, and *WbpL* genes. *B. melitensis* 90 strain lacks the *Lprb/lpcC* gene. The control *B. suis* 1330 and *B. suis* 909 strains lack the *BtpA* gene. *B. abortus* 732 strain lacks the *VceC* gene. The equine *B. melitensis* 68

Table 5 Associated virulence factors in *Brucella* genomes

Virulence factors	Virulence genes and their functions
Lipopolysaccharide (LPS) Outer membrane protein (OMPs)	*AcpXL*, *FabZ*, *Gmd*, *HtrB*, *KdsA*, *KdsB*, *LpsA*, *LpsB*, *LpcC*, *LpxA*, *LpxB*, *LpxC*, *LpxD*, *LpxE*, *ManAoAg*, *ManCoAg*, *Per*, *Pgm*, *Pmm*, *WbdA*, *WbkA*, *WbkB*, *WbkC*, *WboA*, *WbpL*, *WbpZ*, *Wzm*, *Wzt*, *MviN*, *Omp 19.* Penetration, intracellular survival, and immunomodulation. Weak immune response to LPS. Incomplete phagocytosis. Resistance to bactericidal properties of blood. Decreased natural and adaptive immunity. Allergen. Weak inducer of anti-inflammatory cytokines. Inhibition of phagosome-lysosome fusion. Protection from the complement system. Inhibition of intestinal protease.
Type IV secretion system (T4SS)	*VirB1- 12*, effectors *VirB- VceA*, *VceC*, *BPE005*, *BPE123*, *BPE043*, *BPE275*, *SepA*, *BspA*, *BspB*, *BspC*, *BspE*, *BspF* Provides targeted intracellular traffic to the replicative niche. Survival and persistence of *Brucella* inside phagocytes.
Immunoevasion of TIR domain-containing protein	*BtpA*, *BtpB* Blocking *Brucella* recognition by Toll-like receptors (TLR) of phagocytes and inhibiting the intensity of immune-inflammatory reactions. Inhibiting lymphocyte cytotoxicity. Stealthy penetration into the host organism. Evasion of adaptive immunity.
Rab2 interacting conserved protein A	*RicA* Intracellular survival
Cell adhesion molecules	*BmaA*, *BmaC*, *BtaF*
Cyclic β−1,2-glucan (CβG)	*Cgs* Intracellular survival. Formation of *Brucella*-containing vacuoles (BCV). Persistence of *Brucella* inside phagocytes.
The enzyme urease	*Ure.* Protection in an acidic environment. Survival of bacteria when they are localized in the stomach.
Sensory-regulatory adaptation system (BvrS/BvrR)	*BvrS/BvrR.* Control of *Brucella* metabolism in intracellular localization. Adaptation and persistence of *Brucella* inside phagocytes.

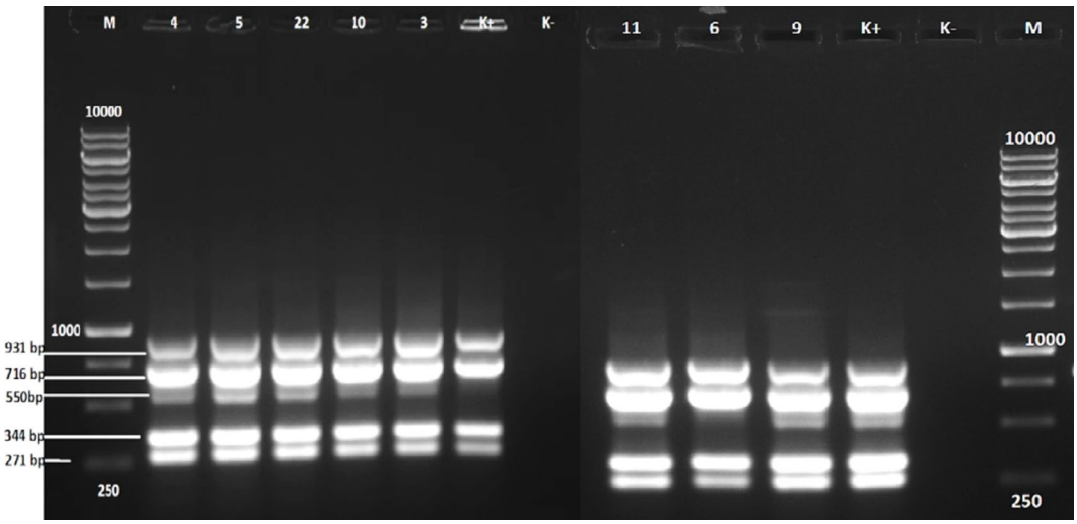

Fig. 5 Multiplex *B. abortus.* Note - M −1Kb O Gene Ruller Marker, K+ - positive control, K- negative control, *Omp* 19–550 bp, *Mvi N*-344 bp, *Man A*-271 bp, *Per A* − 716 bp, *Wbk A* −931 bp, 4-*B. abortus* 732, 5-*B. abortus* Key −44, 22-*B. abortus* B −1, 10-*B. abortus* 487, 3-*B. abortus* 48, 11-*B. abortus* M17, 6-*B. abortus* Key −134, 9 - *B. abortus* 46 2 - *B. abortus 4556*

strain lacks the *BspC* gene. *B. abortus* 732 and 44 strains lack the *Bmac* gene. The *BmaA* gene is present only in *B. suis* 1330 and *B. suis* 909 strains; the *BtaF* gene is present in all *B. suis* strains, but these genes are absent from other strains.

cgSNP analysis identified 7 mutations out of 10 virulence genes: V*ceC*, *BPE275*, *BspB*, *Omp19*, *Ure*, *MviN*,

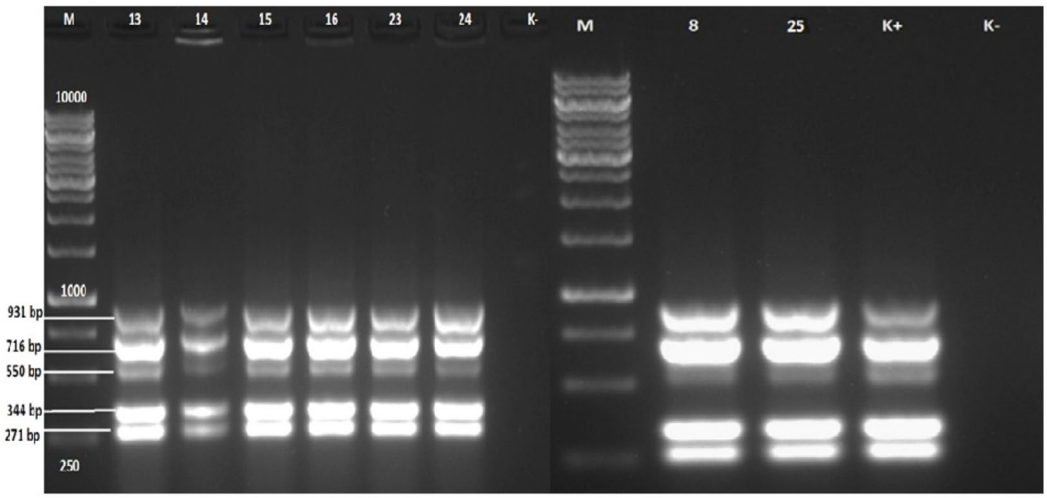

Fig. 6 Multiplex *B. melitensis.* Note - M − 1Kb O Gene Ruller Marker, K+ - positive control, K- negative control, *Omp* 19–550 bp, *Mvi N*-344 bp, *Man A*-271 bp, *Per A* − 716 bp, *Wbk A* − 931 bp, 13-*B. melitensis* 90 Дж, 14-*B. melitensis* 68, 15-*B. melitensis* 3101, 16-*B. melitensis* Key − 41, 23-*B. melitensis* 93, 24-*B. melitensis* BT 2024, 8-*B. melitensis* 499, 25-*B. melitensis* 4

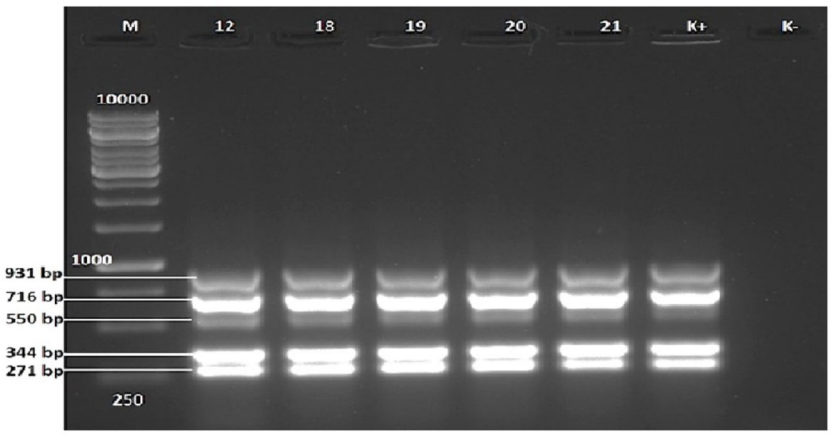

Fig. 7 Multiplex *B. ovis*, *B.suis*, *B.canis.* Note - M − 1Kb O Gene Ruller Marker, K+- positive control, K- negative control, *Omp* 19–550 bp, *Mvi N*-344 bp, *Man A*-271 bp, *Per A* − 716 bp, *Wbk A* − 931 bp, *12-B. suis* 909, 18-*B. ovis* 873, 19-*B. suis* 1330, 20-*B. suis* 4, 21-*B. canis* 1066

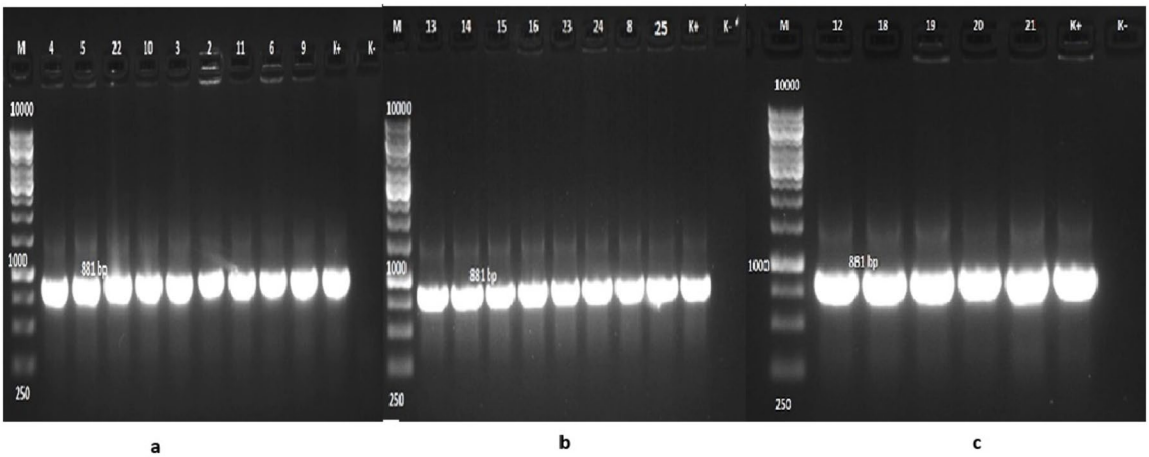

Fig. 8 *VirB* a– *B. abortus*; b– *B. melitensis*; c - *B. ovis*, *B.suis*, *B.canis.* Note - M − 1Kb O Gene Ruller Marker, K+- positive control, K- negative control, *VirB*- 881 bp, 4-*B. abortus* 732, 5-*B. abortus* Key − 44, 22-*B. abortus* B − 1, 10-*B. abortus* 487, 3-*B. abortus* 48, 2 - *B. abortus* 4556,11-*B. abortus* M17, 6-*B. abortus* Key − 134, 9 - *B. abortus* 46, 13-*B. melitensis* 90 Дж, 14-*B. melitensis* 68, 15-*B. melitensis* 3101, 16-*B. melitensis* Key − 41, 23-*B. melitensis* 93, 24-*B. melitensis* BT 2024, 8-*B. melitensis* 499, 25-*B. melitensis* 4, 2-*B. suis* 909, 18-*B. ovis* 873, 19-*B. suis* 1330, 20-*B. suis* 4, 21-*B. canis* 1066

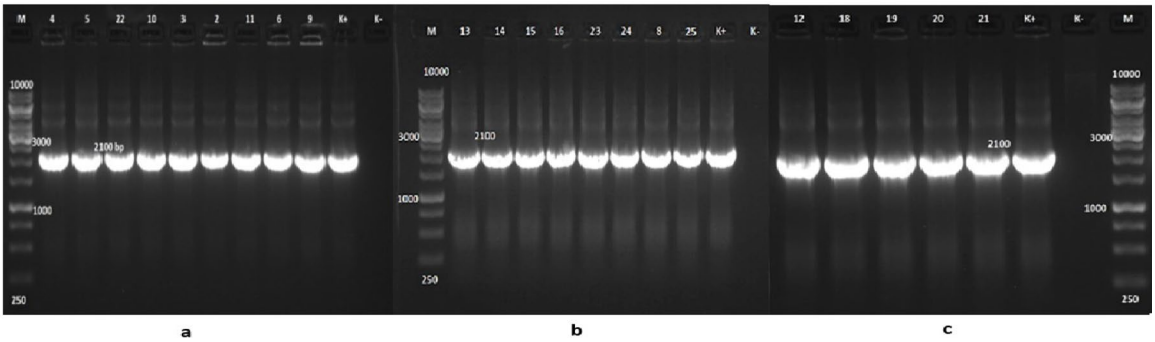

Fig. 9 *Ure* a– *B. abortus*; b– *B. melitensis*; c - *B. ovis*, *B.suis*, *B.canis*. Note - M −1Kb O Gene Ruller Marker, K+- positive control, K- negative control, *Ure* −2100 bp, 4-*B. abortus* 732, 5-*B. abortus* Key −44, 22-*B. abortus* B −1, 10-*B. abortus* 487, 3-*B. abortus* 48, 2 - *B. abortus* 4556,11-*B. abortus* M17, 6-*B. abortus* Key −134, 9 - *B. abortus* 46, 13-*B. melitensis* 90 Дж, 14-*B. melitensis* 68, 15-*B. melitensis* 3101, 16-*B. melitensis* Key −41, 23-*B. melitensis* 93, 24-*B. melitensis* BT 2024, 8-*B. melitensis* 499, 12- *B. melitensis* 909, 25-*B. melitensis* 4, 12-*B. suis* 909, 18-*B. ovis* 873, 19-*B. suis* 1330, 20-*B. suis* 4, 21-*B. canis* 1066

PrpA, *BetB*, *PerA*, and *ManA*, which resulted in amino acid changes in the coding sequences of these virulence genes.

WGS and pan-genome analysis reveal viruloma variations that may have clinical implications for improving disease management and prevention in humans and animals.

For example, 1. The study of virulence genes helps identify targets for antibiotics since inhibition of the *VirB* secretion system reduces *Brucella*'s ability to infect cells (Ponomarenko et al. 2020).

2. Genetic analysis of strains helps track the routes of infection and identify new highly pathogenic strains. This information is necessary for deciding on herd health methods to eradicate the herd or destroy only positively reacting animals.

3. Attenuated or deletion mutants of virulence genes can be used as live vaccines since the Δ*virB* mutant has reduced virulence but retains immunogenicity (Yang et al. 2013; Gheibi et al. 2018).

4. Genetic markers of virulence allow the development of PCR tests for rapid and accurate diagnosis of brucellosis. Examples of target genes: *BCSP31*, *IS711*, *omp25*, *BMEI1162* (Sharova 2001; Kulakov et al. 2009; Kasyan 2017). Moreover, PCR test systems differentiate an animal with brucellosis from a vaccinated one.

To identify mutations of virulence genes, we chose whole-genome sequencing-based single-nucleotide polymorphism (WGS-SNP) analysis because SNP-based typing has a higher resolution than cgMLST analysis of core genome sequences and is suitable for very similar samples, such as the *Brucella* genus, which is characterized by a highly conserved genome. The pan-genome SNP-based phylogeny covers all genome regions, including intergenic regions, and hence covers more areas of the genome than MLST or core genome phylogeny. The reliability and reproducibility of SNP analysis were further demonstrated in the study by Janowicz et al. (Janowicz et al. 2018), who indicated that SNP analysis performed better than MLVA-16 and cgMLST.

Thus, virulence factors may accompany each stage of the brucellosis infection process, and an isolate has multiple virulence factors that influence the dissemination of the bacterium in the host. Characterization of bacterial enzymes and cell wall genes associated with virulence to assess the pathogenicity of *Brucella* is critical for epidemiological studies, outbreak management, and control of brucellosis through eradication programs.

Conclusions

Our study conducted whole-genome sequencing of 21 *Brucella* strains isolated from animals in the Republic of Kazakhstan in 2023–2024 to identify bacterial virulence factors. Then, using single-nucleotide polymorphism analysis, changes were identified in 7 of 10 significant virulence genes. This type of research is being conducted for the first time in the country. It is intended for epidemiological studies in a country endemic to brucellosis, with the aim of eliminating and preventing the disease in animals and humans.

Table 6 SNP mutation analysis in virulence genes of *Brucella* isolates

Gene	Strain ID	Brucella species	Chromosome	Place of mutation	SNP mutation	Amino acid change
PerA	Ba134	*B. abortus*	NZ_KB850274.1	1.062.358	G to A	Leu to Phe
VceC	Bo873	*B. ovis*	NC_009504.1	118.418	G to A	Gly to Asp
BetB	Ba93	*B. melitensis*	NC_003317.1	1.435.747	C to T	Pro to Ser
	Bm4	*B. melitensis*	NC_003317.1	1.435.747	C to T	Pro to Ser
	Bm16M	*B. melitensis*	NC_003317.1	1.435.747	C to T	Pro to Ser
	Bm41	*B. melitensis*	NC_003317.1	1.435.747	C to T	Pro to Ser
	Bm68	*B. melitensis*	NC_003317.1	1.435.747	C to T	Pro to Ser
	Bm499	*B. melitensis*	NC_003317.1	1.435.747	C to T	Pro to Ser
	Bm3101	*B. melitensis*	NC_003317.1	1.435.747	C to T	Pro to Ser
	BT2024	*B. melitensis*	NC_003317.1	1.435.747	C to T	Pro to Ser
PrpA	Ba93	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
	Bm4	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
	Bm16M	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
	Bm41	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
	Bm68	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
	Bm90Dg	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
	Bm499	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
	Bm3101	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
	BT2024	*B. melitensis*	NC_003317.1	267.539	C to T	Arg to Trp
			NC_003317.1	267.758	C to T	Ala to Val
MviN	Ba93	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
	Bm4	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
	Bm16M	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
	Bm41	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
	Bm68	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
	Bm90Dg	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
	Bm499	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
	Bm3101	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
	BT2024	*B. melitensis*	NC_003317.1	1.845.783	G to A	Ala to Thr
Ure	Ba134	*B. abortus*	NZ_KB5852274.1	1.859.135	G to A	Met to Ile
			NZ_KB5852274.1	1.859.353	C to T	Pro to Leu
	Ba4556	*B. abortus*	NZ_KB5852274.1	824.278	A to G	Lys to Glu
			NZ_KB5852274.1	1.859.135	G to A	Met to Ile
			NZ_KB5852274.1	1.859.353	C to T	Pro to Leu
	Ba46	*B. abortus*	NZ_KB5852274.1	1.859.135	G to A	Met to Ile
			NZ_KB5852274.1	1.859.353	C to T	Pro to Leu
	Ba487	*B. abortus*	NZ_KB5852274.1	1.859.135	G to A	Met to Ile
			NZ_KB5852274.1	1.859.353	C to T	Pro to Leu
	BmB1	*B. abortus*	NZ_KB5852274.1	821.761	C to T	Leu to Phe
			NZ_KB5852274.1	824.278	A to G	Lys to Glu
			NZ_KB5852274.1	1.859.135	G to A	Met to Ile
			NZ_KB5852274.1	1.859.353	C to T	Pro to Leu
	Bm487	*B. abortus*	NZ_KB5852274.1	1.859.135	G to A	Met to Ile
			NZ_KB5852274.1	1.859.353	C to T	Pro to Leu

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Author contributions A.D. conceptualization, writing—original draft, writing—review & editing. S.D. methodology, investigation, writing—review & editing. A.A. methodology, investigation. A.A. investigation, formal analysis. N. K. formal analysis. S. P. data curation, writing—review & editing. All authors have read and approved the manuscript for publication.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical statement The Institutional Animal Care and Use Committee of the Kazakh Research Institute of Animal Husbandry and Forage Production performed animal experiments (№ 3 7.11.2022).

Consent to publish Not applicable.

Competing interests The authors declare no competing interests.

References

Aikimbaev AM, Tuleuov AM, Omasheva GM, Chalgynbaeva AU, Mukhama-Diyanova GS (2021) Features of epidemic manifestations of brucellosis in Kazakhstan. Medicine 2(224):2–10. https://medjournal.kz/index.php/journal/article/view/76/63

Brangsch H, Sandalakis V, Babetsa M, Boukouvala E, Ntoula A, Makridaki E, Christidou A et al (2023) Genotype diversity of brucellosis agents isolated from humans and animals in Greece based on whole-genome sequencing. BMC Infect Dis 23(1):529. https://doi.org/10.1186/s12879-023-08518-z

Chiaromonte F, Yap V, Miller W (2002) Scoring pairwise genomic sequence alignments. Pac Symp Biocomputing 115–126. https://doi.org/10.1142/9789812799623_0012

Dadar M, Alamian S, Brangsch H, Elbadawy M, Elkharsawi AR, Neubauer H, Wareth G (2023) Determination of Virulence-Associated genes and antimicrobial resistance profiles in *Brucella* isolates recovered from humans and animals in Iran using NGS technology. Pathogens 12(1):82. https://doi.org/10.3390/pathogens12010082

Daugaliyeva A, Sultanov A, Usserbayev B, Baramova S, Modesto P, Adambayeva A et al (2018) Genotyping of *Brucella melitensis* and *Brucella abortus* strains in Kazakhstan using MLVA-15. Infect Genet Evol 58:135–144. https://doi.org/10.1016/j.meegid.2017.12.022

Daugaliyeva A, Daugaliyeva S, Kydyr N, Peletto S (2024) Molecular typing methods to isolate *Brucella* spp. From animals: A review. Vet World 17(8):1778–1788. https://doi.org/10.14202/vetworld.2024.1778-1788

Edao BM, Ameni G, Berg S, Tekle M, Whatmore AM, Wood JLN, van Tonder AJ, Ashford RT (2023) Whole genome sequencing of Ethiopian Brucella abortus isolates expands the known diversity of an early branching sub-Saharan African lineage. Front Microbiol 14:1128966. https://doi.org/10.3389/fmicb.2023.1128966eCollection 2023

El-Diasty M, El-Said R, Abdelkhalek A (2021) Seroprevalence and molecular diagnosis of sheep brucellosis in Dakahlia Governorate. Egypt Ger J Vet Res 1(1):34–39. https://doi.org/10.51585/gjvr.2021.0006

FAO (2006) Capacity building for surveillance and control of bovine and caprine brucellosis. https://www.fao.org/4/a0083e/a0083e0a.htm

Gautam SS, Kc R, Leong KW, Mac Aogáin M, O'Toole RF (2019) A step-by-step beginner's protocol for whole genome sequencing of human bacterial pathogens. J Biol Methods 6(1):e110. https://doi.org/10.14440/jbm.2019.276

Gheibi A, Khanahmad H, Kashfi K, Sarmadi M, Khorramizadeh MR (2018) Development of new generation of vaccines for *Brucella abortus*. Heliyon 4(12):e01079. https://doi.org/10.1016/j.heliyon.2018.e01079

Halling SM, Peterson–Burch BD, Bricker BJ, Zuerner RL, Li QZ L.L (2005) Completion of the genome sequence of *Brucella abortus* and comparison to the highly similar genomes of *Brucella melitensis* and *Brucella suis*. J Bacteriol 187(8):2715–2726. https://doi.org/10.1128/JB.187.8.2715-2726.2005

Hernández-Mora G, Chacón-Díaz C, Moreira-Soto A, Barrantes-Granados O, Suárez-Esquivel M, Viquez-Ruiz E, Barquero-Calvo E et al (2023) Virulent *Brucella nosferati* infecting *Desmodus rotundus* has emerging potential due to the broad foraging range of its Bat host for humans and wild and domestic animals. mSphere 8(4):e0006123. https://doi.org/10.1128/msphere.00061-23

Ilyas M, Harpke M, Wareth G (2024) Brucellosis in Iraq: A comprehensive overview of public health and agricultural challenges. German J Microbiol 4(3):10–20. https://doi.org/10.51585/gjm.2024.3.0039

Janowicz A, De Massis F, Ancora M, Cammà C, Patavino C, Battisti A, Prior K et al (2018) Core genome multi locus sequence typing and single nucleotide polymorphism analyses in the epidemiology of *Brucella melitensis* infections. J Clin Microbiol 56(9):e00517–e00518. https://doi.org/10.1128/JCM.00517-18

Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O (2014) Solving the problem of comparing whole bacterial genomes across different sequencing platforms. PLOS ONE. https://doi.org/10.1371/journal.pone.0104984. eCollection 2014

Karakululy R, Dosybaev M, Ryskulova A, Sarsenbaeva M, Moldamyrza S (2023) Epidemiological monitoring of the brucellosis epidemic in the Republic of Kazakhstan over a Five-Year period 2018–2022. Public Health 35–44. https://doi.org/10.24412/1609-8692-2023-3-35-44

Karthik K, Anbazhagan S, Thomas P, Chitra MA, Alagesan Senthilkumar TM, Sridhar R (2021) Genome sequencing and comparative

genomics of Indian isolates of *Brucella melitensis*. Front Microbiol 12:1–11. https://doi.org/10.3389/fmicb.2021.698069

Kasyan J (2017) Development of methodological approaches and diagnostic preparations for determining species and biovars of Brucella based on molecular genetic technologies. https://www.dissercat.com/content/razrabotka-metodicheskikh-podkhodov-i-diagnosticheskikh-preparatov-dlya-opredeleniya-vidov

Khan AU, Melzer F, Sayour AE, Shell WS, Linde J, Abdel-Glil M et al (2021) Whole-Genome sequencing for tracing the genetic diversity of Brucella abortus and Brucella melitensis isolated from livestock in Egypt. Pathogens 10(6):759. https://doi.org/10.3390/pathogens10060759

Kulakov YuK, Zheludkov MM, Tolmacheva TA, Tsirelson LE (2009) PCR method in laboratory diagnosis of brucellosis. Epidemiology 29–33

Kydyshov K, Usenbaev N, Berdiev S, Dzhaparova A, Abidova A, Kebekbaeva N et al (2022a) First record of the human infection of *Brucella melitensis* in Kyrgyzstan: evidence from whole-genome sequencing-based analysis. Infect Dis Poverty 11(1):120. https://doi.org/10.1186/s40249-022-01044-1

Kydyshov K, Usenbaev N, Sharshenbekov A, Aitkuluev N, Abdyraev M, Chegirov S, Kazybaeva J, Brangsch H, Melzer F, Neubauer H et al (2022b) Brucellosis in humans and animals in Kyrgyzstan. Microorganisms 10(7):1293. https://www.mdpi.com/2076-2607/10/7/1293

Laine CG, Johnson VE, Scott HM, Arenas-Gamboa AM (2023) Global estimate of human Brucellosis incidence. Emerg Infect Dis 29. https://doi.org/10.3201/eid2909.230052

Liu B, Zheng D, Jin Q, Chen L, Yang J (2019) VFDB 2019: A comparative pathogenomic platform with an interactive web interface. Nucleic Acids Res 47:D687–D692. https://doi.org/10.1093/nar/gky1080

Liu Zh, Gao L, Wang M, Yuan M, Li Zh (2024) Long ignored but making a comeback: a worldwide epidemiological evolution of human brucellosis. Emerg Microbes Infect 13(1):2290839. https://doi.org/10.1080/22221751.2023.2290839

Liu Zh, Gao L, Wang M, Du S, Yuan M, Li Zh (2025) Preplanned studies: global species/biovars and genotype diversity atlas of *Brucella* spp. — 102 countries, 1923–2020. China CDC Wkly 7(4):144–151. https://doi.org/10.46234/ccdcw2025.023

Mirnejad R, Jazi FM, Mostafaei Sh, Sedighi M (2017) Molecular investigation of virulence factors of *Brucella melitensis* and *Brucella abortus* strains isolated from clinical and non-clinical samples. Microb Pathog 109:8e14. https://doi.org/10.1016/j.micpath.2017.05.019

Papaparaskevas J, Procopiou A, Routsias J, Vrioni G, Tsakris A (2023) Detection of Virulence-Associated genes among Brucella melitensis and Brucella abortus clinical isolates in Greece 2001–2022. Pathogens 12(11):1274. https://doi.org/10.3390/pathogens12111274

Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV (2006) The new global map of human brucellosis. Lancet Infect Dis 6(2):91–99. https://doi.org/10.1016/S1473-3099(06)70382-6

Ponomarenko DG, Sarkisyan NS, Kulichenko AN (2020) Pathogenesis of brucellosis: analysis of the immunopathological concept. J Continuing Med Educ Physicians 9(3):96–105. https://cyberleninka.ru/article/n/patogenez-brutselleza-analiz-immunopatologicheskoy-kontseptsii-material-dlya-podgotovki-lektsii.pdf

Rabinowitz P, Zilberman B, Motro Y, Roberts MC, Greninger A, Nesher L (2021) Whole genome sequence analysis of *Brucella melitensis* phylogeny and virulence factors. Microbiol Res 12(3):698–710. https://doi.org/10.3390/microbiolres12030050

Scholz HC, Nöckler K, Göllner C, Bahn P, Vergnaud G, Tomaso H, Al Dahouk S, Kämpfer P, Cloeckaert A, Maquart M et al (2010) *Brucella inopinata* Sp. nov., isolated from a breast implant infection. Int J Syst Evol Microbiol 60(Pt 4):801–808. https://doi.org/10.1099/ijs.0.011148-0

Sharova I (2001) Improvement of the test system for detection of the causative agent of brucellosis by the PCR method. Dissertation. https://www.dissercat.com/content/sovershenstvovanie-test-sistemy-dlya-vyyavleniya-vozbuditelya-brutselleza-metodom-ptsr

Syrym NS, Yespembetov BA, Sarmykova MK, Konbayeva GM, Koshemetov ZK, Akmatova EK, Bazarbaev M, Siyabekov ST (2019) Reasons behind the epidemiological situation of brucellosis in the Republic of Kazakhstan. Acta Trop 191:98–107. https://www.sciencedirect.com/science/article/abs/pii/S0001706X18312270

Taipova A, Beishova IS, Alikhanov KD, Otarbayev VK, Ulyanov VA, Ginayatov NS, Dushaeva L, Zh (2023) Monitoring of the epizootic situation on animal brucellosis in the Republic of Kazakhstan. Ġylym Žáne Bilim 2(2):161–169. https://doi.org/10.52578/2305-9397-2023-2-2-161-169

Terrestrial Animal Manual (2015) Section 3.8. Caprinae. Chapter 3.8.7. Ovine epididymitis (*Brucella ovis*). https://rr-europe.woah.org/app/uploads/2021/08/3-7-8.pdf. Accessed May 2015

Terrestrial Animal Manual (2022) Section 3.1. Diseases common to all animal species. Chapter 3.1.4. Brucellosis (*Brucella abortus, B. melitensis* and *B. suis*). https://rr-europe.woah.org/app/uploads/2021/08/3-1-4-brc_.pdf. Accessed May 2022

Urazaeva AB, Bekenov ZE, Urazaeva ST (2018) Epidemic potential of brucellosis in the Aktobe region. Medicine 11(197):71–77. https://www.medzdrav.kz/images/magazine/medecine/2018/2018-11/13.pdf

WHO (2020) Brucellosis. https://www.who.int/ru/news-room/fact-sheets/detail/brucellosis

Xue H, Li J, Ma L, Yang X, Ren L, Zhao Zh, Wang J et al (2023) Seroprevalence and molecular characterization of Brucella abortus from the Himalayan Marmot in Qinghai, China. Infect Drug Resist 16:7721–7734. https://doi.org/10.2147/IDR.S436950. eCollection 2023

Yang X, Skyberg JA, Cao L, Clapp B, Pascual TT D.W (2013) Progress in *Brucella* vaccine development. Front Biol 8:60–77. https://doi.org/10.1007/s11515-012-1196-0

Yang X, Piao D, Mao L, Pang B, Zhao H, Tian G et al (2020) Whole-genome sequencing of rough *Brucella melitensis* in China provides insights into its genetic features. Emerg Microbes Infect 9(1):2147–2156. https://doi.org/10.1080/22221751.2020.1824549

Yespembetov BA, Syrym NS, Zinina NN et al (2019) Phenotypic and genotypic characteristics of *Brucella* isolates from the Republic of Kazakhstan. Trop Anim Health Prod. 51:2361–2370. https://link.springer.com/article/10.1007/s11250-019-01941-y