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Characterization of *Brucella* spp. circulating in industrial dairy cattle farms in Iran: a field study 2016 - 2023

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Abstract

Bovine brucellosis, an infectious disease transmitted by *Brucella melitensis* and *Brucella abortus*, presents a significant zoonotic risk for agricultural economics and animal health. The primary objective of this study was to present a comprehensive understanding of the prevalence and features of *Brucella* strains within the industrial dairy farming sector in Iran. Rose Bengal plate test, standard agglutination test, and indirect enzyme linked immunosorbent assay tests were used to confirm all seropositive animals. A total number of 1,311 bovine samples from seropositive animals including were collected from 224 farms in 21 provinces of different regions of Iran and examined. The discovered *Brucella* isolates were phenotyped and molecularly characterized. The isolates were all *B. abortus* or *B. melitensis*. Bacteria analysis revealed that 70.53% of seropositive farms were tested positive for *Brucella* strains, predominantly *B. melitensis* biovar 1 (43.42%) and *B. abortus* biovar 3 (27.11%). Geographical distribution revealed that *B. melitensis* biovar 1 was the most common in dairy cow farms (16 provinces), followed by *B. abortus* biovar 3 (six provinces). Also, the prevalence of *B. melitensis* biovar 2, *B. melitensis* biovar 3, *B. abortus* biovar 1, *B. abortus* biovar 2 and RB51 vaccine were restricted to certain provinces. AMOS (*abortus melitensis ovis suis*)-polymerase chain reaction and Bruce-ladder PCR confirmed species identification. These results highlighted the complexity of bovine brucellosis in Iran and illustrated that *B. melitensis* was spread from small ruminants to cattle. This study provided important epidemiological insights for targeting future brucellosis control programs in the Iranian dairy farms.

Introduction

Zoonotic infection of brucellosis, is caused by various bacterial species from the *Brucella* genus, predominantly *Brucella abortus*, *B. melitensis*, and *B. suis*. This disease is closely linked to development of agricultural communities where animal husbandry plays a vital role. *B. abortus* is facultative intracellular pathogen that is capable of causing long-lasting infections in animals and has been found in various livestock species. More specifically, *B. abortus* is frequently found in cows which act as its primary host and cause bovine brucellosis.[1,2] This bacterial infection is a major concern for both farmers and policy makers because of its negative impact on animal health and the agricultural industry as a whole.[3,4] Bovine brucellosis, a highly contagious and economically significant disease, continues to pose a significant threat to livestock in Iran. In recent years, there has been an alarming increase in bovine brucellosis cases in Iran. This increase can be attributed to several factors including ineffective control strategies, limited public awareness and inadequate veterinary services in certain regions.[5] Also, the practice of diverse agriculture, which includes the keeping of buffalo, cows, goats, and sheep has resulted in a higher risk of brucellosis. In this scenario, small ruminants serve as primary hosts for *B. melitensis*, while cattle serve as secondary hosts.[6] In this regard, many farmers face significant losses as infected cattle experience reduced milk production, reproductive problems and even death. In an infected herd, some animals show symptoms while others remain latent. The most common sign is miscarriage as well as other signs such as frequent and unsuccessful inseminations, reduced milk production, retained placenta, metritis, arthritis and orchitis.[7] In Iran, the key causative agents responsible for bovine brucellosis have been reported as different types of *B. abortus* and

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occasionally, *B. melitensis*, that play a significant role in spread of the disease among cattle herds.[8,9] Furthermore, a different investigation found that *B. melitensis* and *B. abortus* were prevalent in cattle.[10] As a result, it is crucial to accurately determine the specific *Brucella* species and biotypes in order to effectively implement control and eradication initiatives. This serves to clearly indicate the prevailing *Brucella* species and biotypes in a particular region or country as well as monitoring vaccine strains used during vaccination campaigns. Also, this information can aid in tracking and managing newly introduced strains and it is essential for accurately assessing the epidemiological situation of livestock herds and countries.

The evaluation of *Brucella*-specific genes plays a crucial role in assessing the efficacy of PCR-based detection methods for *Brucella* detection. Currently, there exist specialized primers and probes that are tailored for the purpose of detecting *Brucella*. Numerous researches have been conducted, primarily centering around the genes *IS711*, *16S rRNA*, *bcsp31*, *BMEI1162*, *BMEII0466*, *eryC*, *alkB*, and *per*.[11] The *IS711* gene, along with other genes, serves as a valuable molecular marker in the diagnosis and characterization of *Brucella.* However, the *IS711* gene is highly specific to the *Brucella* genus and is present in multiple copies within the genome of *Brucella* species. Different *Brucella* species possess distinct patterns and numbers of *IS711* copies allowing for the differentiation of various *Brucella* strains. These tools contribute not only to accurate and rapid identification of *Brucella* species but also play a crucial role in understanding the epidemiology, evolution and dynamics of brucellosis.[8,12] On the other hand, AMOS (*abortus melitensis ovis suis*)- and Bruce-ladder polymerase chain reaction (PCR) are molecular based techniques that leverage the variability in tandem repeat regions of the *Brucella* genome to differentiate between strains and species providing valuable information for epidemiological studies and disease tracking. Bricker and Halling developed the AMOS PCR assay to differentiate four *Brucella* species including *B. abortus*, *B. melitensis*, *Brucella ovis*, and *B. suis*.[13]

The primary aim of this study was to distinguish *Brucella* isolates in industrial dairy cattle farms through the utilization of both traditional methods and molecular approaches. Additionally, we aimed to monitor the occurrence of common biotypes *Brucella* strains, and vaccine strains in the field.

Materials and Methods

Ethical statement. All dairy cattle in this investigation were treated considering to ethical standards for field study approved by the Iranian Veterinary Organization (Tehran, Iran). Approval for the research was granted by Ethics Committee for Health Research at Razi Vaccine and Serum Research Institute in June 2022 (Reference: IR.RVSRI.REC. 1402.002). The animals were slaughtered according to the test-and-slaughter programs of Iranian Veterinary Organization. The purpose of the study was explained to the dairy farmers and their consent was obtained.

Study area. This study was performed from June 2016 to June 2023, on 364 milk samples, 907 bovine lymph nodes, nine samples of abomasum content, and 31 aborted fetuses to evaluate the presence of *Brucella* spp. in seropositive dairy cattle that tested by Rose Bengal plate test (RBPT), standard agglutination test (SAT), and indirect enzyme linked immunosorbent assay tests (I-ELISA) methods. A variety of specimens were collected from 21 provinces located across different regions of Iran (Tehran, East Azerbaijan, Alborz, West Azerbaijan, Chaharmahal and Bakhtiari, Ardabil, Kohgiluyeh and Boyer-Ahmad, Kermanshah, Markazi, Lorestan, Isfahan, Qazvin, Hamadan, Ilam, Qom, Zanjan, Semnan, Yazd, Fars, Kerman and Mazandaran). These regions are critical for the dairy industry and boast a vibrant agricultural sphere.

Sample collection. Sampling was conducted on available seropositive lactating cattle within the herds of industrial dairy cattle farms using RBPT, Wright, and I-ELISA tests. A total of 1,311 specimens were sampled from 224 farms (Table 1). The milk and lymph node samples from different seropositive farms were collected by the veterinary organization of each province and were sent to department of Brucellosis, Razi Vaccine and Serum Research Institute for analysis (Karaj, Iran). Lymph node specimens were collected from supra-mammary and retropharyngeal regions of slaughtered animals in sterile plastic bags and placed on ice and immediately transported to the laboratory. These preserved samples were intended for subsequent *Brucella* culture and isolation procedures.

Serological analysis. The sera of 1,311 blood samples were isolated using a centrifugation process at 3,000 rpm for 5 min. The serum from each sample was subjected to testing using RBPT, SAT (Razi Vaccine and Serum Research Institute, Karaj, Iran), and I-ELISA methods (IDEXX, Montpellier, France). The I-ELISA test was conducted according to the guidelines specified by the manufacturer, available at this link: (https://www.id-vet.com/produit/id-screen-brucellosis-serum-indirect-multi-species/). Serum specimens were deemed positive if the titers were equal to or exceeded 1:80. Dairy cattle farm are known as reactors when their serum of blood in official serological tests was positive.

***Brucella* isolation.** Bacteriological examinations were carried out within the protective environment of safety hoods. Bacterial culture was performed on samples of milk, aborted fetal organs and abomasum content of dairy cattle. The initial isolation of *Brucella* spp. was involved by inoculating the clinical samples onto a specialized medium of *Brucella* Selective Supplement (Oxoid, Basingstoke, UK).

Table 1. The properties of samples tested for *Brucella* spp. by bacterial culture.

Provinces (Number of farms)	Samples (n)
West Azerbaijan (2)	Lymph nodes (3)
East Azerbaijan (11)	Lymph nodes (20)
Ardabil (9)	Lymph nodes (7); Milk (10)
Kohgiluyeh and Boyer-Ahmad (1)	Lymph nodes (2)
Kermanshah (2)	Lymph nodes (10); Milk (25)
Markazi (1)	Lymph nodes (70)
Hamadan (3)	Lymph nodes (49)
Qazvin (28)	Lymph nodes (79); Milk (30)
Ilam (1)	Lymph node (1)
Alborz (30)	Lymph nodes (106); Milk (5)
Lorestan (3)	Lymph nodes (23); Milk (20)
Qom (36)	Lymph nodes (101); Milk (15)
Tehran (31)	Lymph nodes (166); Milk (199)
Isfahan (15)	Lymph nodes (50); Milk (3); Fetuses (15)
Chaharmahal and Bakhtiari (4)	Lymph nodes (45)
Zanjan (1)	Milk (2)
Semnan (6)	Lymph nodes (14); Milk (20)
Yazd (5)	Lymph nodes (12); Milk (1); Fetuses (2)
Fars (26)	Lymph nodes (114); Milk (10); Fetuses (14); Abomasum content (8)
Kerman (8)	Lymph nodes (35); Milk (24)
Mazandaran (1)	Abomasum content (1)
Total (224)	Lymph nodes (907); Milk (364); Fetuses (31); Abomasum content (9)

This supplement contained various components such as cycloheximide (50.00 mg), nystatin (50.00 IU), bacitracin (12.50 IU), polymyxin B (2.50 IU), vancomycin (10.00 mg) and nalidixic acid (2.50 mg). Additionally, the culture medium used was comprised of inactivated horse serum (5.00%) in *Brucella* agar (HiMedia, Thane, India). These cultures were then incubated for a period of 10 days maintaining a temperature of 37.00 ˚C while also sustaining an atmosphere of 10.00% CO_2.[8] The milk samples underwent a 15-min centrifugation at 3,500 rpm followed by the cultivation of both sediments and the creamy upper layer. If no visible growth occurred after a 14-day incubation, the bacterial cultures were disposed. Distinct colonies representing *Brucella* spp. were then transferred to new culture media of *Brucella* agar for detailed identification and biotyping analysis.

Biotyping. The classic method of biotyping followed the protocol outlined previously.[8] At this facility, standard diagnostic procedures utilized *Brucella* monospecific antisera A and M, along with the *Brucella* reference phage from Tbilisi, Izzatnagar. A diverse range of biotyping tests were conducted including assessments for H_2S production, dependence to CO_2, agglutination using specific *Brucella* antisera, agglutination with acriflavine, growth in media with thionine and basic fuchsin. Interpretation of the test results was in accordance with the guidelines provided in the manual of World Organization for Animal Health.

Molecular typing. The genomic DNA was obtained by subjecting a small amount of bacterial material suspended in 300 µL of high-quality water for molecular biology to a heat treatment at 100 ˚C for a duration of 15 min.[14] The mixture was thoroughly mixed using a vortex and then subjected to centrifugation at a force of 13,000 *g* for 5 min. Following this, the liquid portion containing DNA was gathered and kept at a temperature of – 20.00 ˚C until it was needed for subsequent analysis.[8] The DNA isolated underwent a PCR assay utilizing the *IS711* marker to detect the presence of *Brucella* spp (Table 2).[15] Species-level molecular characterization was additionally carried out through a composite Bruce-ladder PCR (Bio-Rad Laboratories, Hercules, USA) utilizing the subsequent parameters (Table 2): Step 1 (first denaturation): 95.00 ˚C for 5 min, step 2 (second denaturation): 95.00 ˚C for 30 sec, step 3 (annealing): 56.00 ˚C for 90 sec, step 4 (first extension): 72.00 ˚C for 3 min, step 5 (final extension): 72.00 ˚C for 10 min. Step 2, 3, and 4 were reiterated for a total of 30 cycles.[16] The resulting PCR products were resolved through electrophoresis (Bio-Rad Laboratories), utilizing a gel made from 1.50% agarose.

Results

A total number of 420 *Brucella* strains were extracted from a diverse range of seropositive sources originating from 158 out of 224 distinct farms (70.53%). These sources encompassed lymph nodes (239 isolates), milk (161 isolates), aborted fetuses (14 isolates), and abomasum contents (six isolates). These isolated bacteria exhibited typical phenotypic features commonly associated with the *Brucella* genus. The growth behaviour of all strains was observed in an environment with 10.00% carbon dioxide (CO_2) following an incubation period of 3 to 5 days at a temperature of 37.00 ˚C. Gram-negative characteristics were evident in the isolated bacteria and

they formed small colonies with a distinctive translucent, honey-colored and glossy appearance, featuring a smooth surface. The characterization of the isolates extended to the biovar level with the identification of each isolate being confirmed to the species or vaccine level using AMOS PCR and Bruce-ladder techniques. These isolates represented either *B. melitensis*, *B. abortus*, or the *B. abortus* vaccine strain RB51.

Brucella species/biovars geographical distribution. It has been shown that *B. melitensis* biovar 1 was the most prevalent biovar in dairy cattle farms of 16 provinces in Iran (Alborz, Tehran, Fars, Qom, Kerman, Isfahan, Semnan, Qazvin, Lorestan, Chaharmahal and Bakhtiari, Zanjan, Hamedan, Kermanshah, Kohgiluyeh and Boyer-Ahmad, West Azerbaijan and, East Azerbaijan). The *B. melitensis* biovar 2 was only isolated in Kerman province, while *B. melitensis* biovar 3 was isolated from the four provinces of Fars, Kerman, Isfahan and Ardabil. *B. abortus* biovar 1 was identified from samples the three provinces of Fars, Qom and Yazd. *B. abortus* biovar 2 was only isolated in Yazd Province, and *B. abortus* biovar 3 was identified in six provinces of Tehran, Fars, Qom, Isfahan, Lorestan, and Hamedan. Also, the strain of RB51 vaccine was detected from provinces of Kermanshah and Alborz (Fig. 1).

***Brucella abortus*.** A total number of 213 different strains of *B. abortus* were found across 61 farms spanning various provinces, including Tehran, Fars, Yazd, Qom, Isfahan, Lorestan, and Hamedan. The majority of *B. abortus* cases (105 cases) were linked to lymph nodes with 86 cases originating from milk samples, six cases from abomasum content, and 14 cases from aborted fetuses.

The biotyping results were revealed with the presence of *B. abortus* biovar 1 (5 cases), biovar 2 (1 case), and biovar 3 (205 cases). The final two cases isolated from lymph node samples represented the strain of *B. abortus* RB51 vaccine according to Bruce-ladder typing. Notably, the AMOS PCR detected the *B. abortus* specific band (498 bp) exclusively in biovar 1 and 2 isolates, in line with its capability to identify biovars 1, 2, and 4 (Fig. 2A).[16] It is worth noting that all isolates were confirmed as *B. abortus* through the Bruce ladder PCR, revealing PCR products with sizes of 152, 450, 587, 794, and 1,682 bp (Fig. 2B).

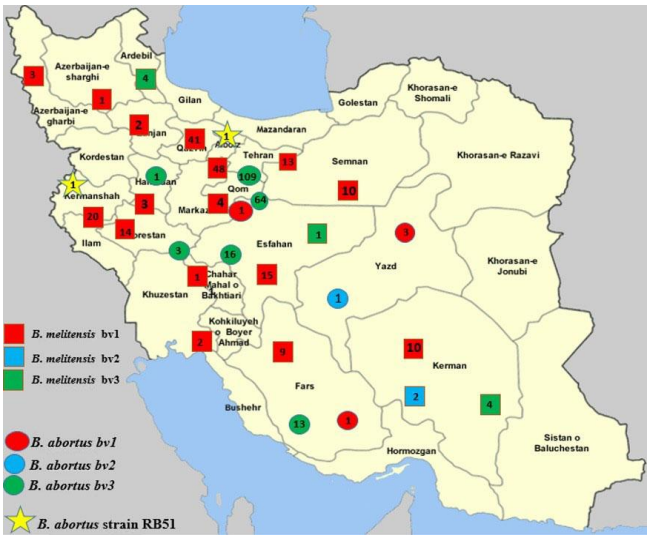

Fig. 1. Geographic distribution of *Brucella* species/biovars from industrial dairy cattle in Iran. The numbers inside the boxes showed the *Brucella* biovars frequencies.

Table 2. Primer sets and expected amplicon sizes specific to different *Brucella* species.

Strain amplicon	Primer set	Primer sequence (5-3')	DNA target	Size (bp)	References
AMOS PCR	IS711 AB	TGCCGATCACTTTCAAGGGCCTTCAT GACGAACGGAATTTTTCCAATCCC	IS711	498	15
AMOS PCR	IS711 BM	TGCCGATCACTTTCAAGGGCCTTCAT AAATCGCGTCCTTGCTGGTCTGA	IS711	731	15
AMOS PCR	IS711 *B. ovis*	TGCCGATCACTTTCAAGGGCCTTCAT CGGGTTCTGGCACCATCGTCG	IS711	976	15
AMOS PCR	IS711 *B. suis*	TGCCGATCACTTTCAAGGGCCTTCAT GCGCGGTTTTCTGAAGGTTCAGG	IS711	285	15
Bruce-ladder PCR	BMEI0998f BMEI0997r	ATC CTA TTG CCC CGATAA GG GCT TCG CAT TTT CACTGT AGC	Glycosyltransferase, gene wboA	1,682	16
Bruce-ladder PCR	BMEI0535f BMEI0536r	GCG CAT TCT TCG GTTATG AA CGC AGG CGA AAA CAGCTA TAA	Immunodominant antigen, gene bp26	450	16
Bruce-ladder PCR	BMEII0843f BMEII0844r	TTT ACA CAG GCA ATCCAG CA GCG TCC AGT TGT TGTTGA TG	Outer membrane protein, gene omp31	1071	16
Bruce-ladder PCR	BMEI1436f BMEI1435r	ACG CAG ACG ACC TTCGGTAT TTT ATC CAT CGC CCTGTCAC	Polysaccharide deacetylase	794	16
Bruce-ladder PCR	BMEII0428f BMEII0428r	GCC GCT ATT ATG TGGACT GG AAT GAC TTC ACG GTCGTT CG	Erythritol catabolism, gene eryC (D-Erythrulose -1-phosphatedehydrogenase)	587	16
Bruce-ladder PCR	BR0953f BR0953r	GGA ACA CTA CGC CACCTT GT GAT GGA GCA AAC GCTGAA G	ABC transporter binding protein	272	16
Bruce-ladder PCR	BMEI0752f BMEI0752r	CAG GCA AAC CCT CAG AAG C GAT GTG GTA ACG CAC ACC AA	Ribosomal protein S12, gene *rpsL*	218	16
Bruce-ladder PCR	BMEII0987f BMEII0987r	CGC AGA CAG TGA CCATCA AA GTA TTC AGC CCC CGTTAC CT	Transcriptional regulator, CRP family	152	16

***Brucella melitensis*.** In all 207 strains of *B. melitensis* were isolated from 97 farms including case series in lymph nodes (132 cases), and milk (75 cases). Isolates represented all three biovars but with *B. melitensis* biovar 1 (196 cases) more common than *B. melitensis* biovars 2 (two cases) and *B. melitensis* biovars 3 (nine cases). All other isolates were confirmed as wild type *B. melitensis* by both Bruce-ladder PCR with products of 1,682, 1,071,794, 587, 450, and 152 bp and AMOS-PCR with a PCR product of 731 bp in size.

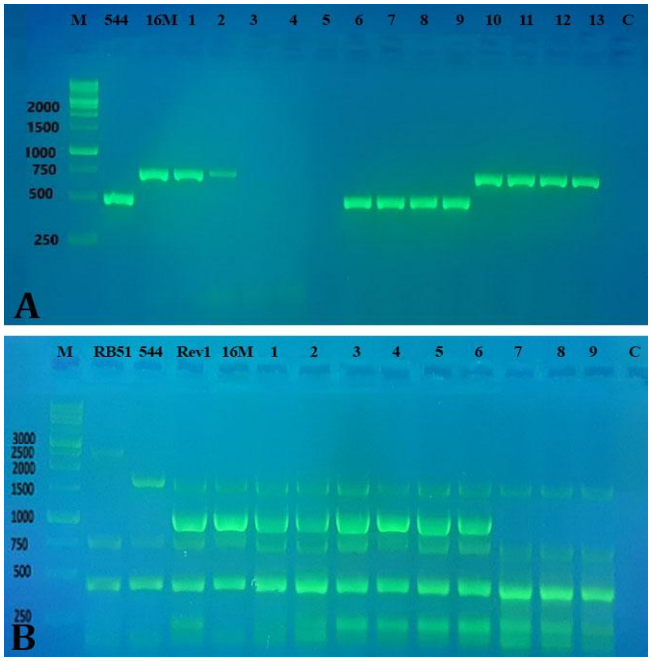

Fig. 2. A) AMOS-PCR. Lane M: molecular marker 1,000 base pairs (bp), Lanes 1, 2, and 10-13: *Brucella melitensis* (band 731 bp), Lanes 3,4 and 5: *Brucella abortus* biovar 3 which does not amplify in AMOS PCR, Lane C: negative control, Lane 544: *Brucella abortus* reference strain 544, Lane 16 M: *Brucella melitensis* reference strain 16 M, Lanes 6-9 with band 498 bp *Brucella abortus* strains isolated from the field in livestock samples. **B)** Bruce-ladder PCR. Lane M: 1.00 kb molecular marker, Lane RB51: Bovine vaccine, Lane 544: Reference strain of *Brucella abortus* (544), Lane Rev1: Sheep vaccinal strain, Lane 16 M: *Brucella melitensis* reference strain 16 M, Lanes 1 and 2 *Brucella melitensis*. Lanes 3 to 6: *Brucella melitensis* vaccine strain, Lanes 7-9: *Brucella abortus* field strain. Lane C: negative control.

Discussion

Given the widespread occurrence of brucellosis in Iran, it is essential to have a deep understanding of the clinical and epidemiological aspects of virulent *Brucella* species. This knowledge is critical for enhancing the accuracy of diagnosis, prevention and control measures. The most reliable method for diagnosing brucellosis in animals remains bacterial culture and isolation followed by comprehensive bacteriological testing and biotyping.[4,17] In numerous studies conducted in Iran, *Brucella* infection has been primarily reported through PCR and serology tests. However, there has been a limited focus on directly identifying the presence of *Brucella* species and their biovars in recent research.[4] To address this gap, we conducted a thorough analysis using a combination of bacteriological and molecular techniques. Our study aimed to provide a comprehensive characterization of *Brucella* biodiversity within the industrial dairy cattle farms affected by the infection in Iran. The results we presented here represented a passive surveillance effort spanning seven years. These findings significantly contributed to our understanding of the specific *Brucella* species and biovars currently linked to the disease in industrial dairy cattle farms of Iran, clearly highlighting a notable prevalence of both *B. melitensis* and *B. abortus*. When focusing on dairy cattle farms, our observations indicated that *B. melitensis* was predominantly associated with cattle in different provinces of Iran. In contrast, *B. abortus* was prevalent in some provinces in dairy cattle farms. These findings were consistent with the growing trend of *B. melitensis* isolation, particularly in cattle, especially in regions such as Africa and the Middle East,[10,18-20] and with previous observations in Iran.[4,9] In this study, bovine brucellosis primarily seemed to be linked to *B. melitensis*, affecting 43.50% of infected farms. There was a lesser prevalence of *B. abortus*, impacting 27.20% of these farms. This pattern was consistent with the belief that *B. melitensis* is present in dairy cattle.[21-23] Despite the small ruminant vaccination program, our findings indicated that *B. melitensis* has spilled over from these ruminants to cattle. Specifically, in the Iran, cattle must be regarded as a potential reservoir of both *B. abortus* and *B. melitensis* for transmission to human. According to our results, *B. melitensis* biovar 1 and *B. abortus* biovar 3 was the biovar predominantly isolated from dairy cattle with rarer isolation of *B. melitensis* biovars 2, *B. melitensis* biovars 3, *B. abortus* biovar 2 and *B. abortus* biovar 1. These findings were in agreement with previous research conducted in various regions across the globe, indicating that *B. melitensis* biovars 1 are extensively prevalent in cattle.[18,21,24]

According to our findings, *B. melitensis* biovars 1 (94.74%), 2 (1.00%) and 3 (4.33%) were the species that have been isolated in 97 dairy cattle farms from lymph node and milk samples. *B.* melitensis biovar 1 initially emerged after being identified in a sheep located in Isfahan (the center of Iran). Subsequently, it spread to various regions within Iran, causing infections in not only sheep and goats, but also in cattle, camels, dogs and humans.[4,9] The investigation performed by Zowghi *et al*., involving the culture of animal fetuses, placentas, lymph nodes, milk, vaginal swabs and human bone marrow and blood identified a total number of 2,413 strains of *B. melitensis* that underwent the process of identification and revealed that *B. melitensis* strains were isolated from cattle, sheep, goats, camels, dogs, and humans in Iran.[9] The

B. melitensis biovar 1 was also isolated in cows from Egypt,[20] South Africa,[20] Azerbaijan,[21] Kenya,[22] Spain,[24] China,[25] Kuwait,[26] Syria,[27] Algeria,[28] and Uganda.[29] The present study also showed the common prevalence of *B. melitensis* in dairy cattle farms of Iran.

The *B. abortus* biovar 3 was emerged as the dominant strain in bovines consistent with a prior epidemiological investigations conducted in Iran identifying this particular biovar as the primary and most virulent variant affecting cattle.[9] Nevertheless, our findings indicated that *B. abortus* biovar 3 was similarly implicated in causing abortions among dairy cows in the provinces of Fars and Isfahan much like documented occurrences in Europe, Kenya, China and Türkiye.[30] Based on our findings, the occurrence of *B. melitensis* biovar 2 was solely documented in Kerman bovine population. Despite its predominance in China,[31] this particular biovar appears to exhibit a reduced presence in regions encompassing the Middle East and Mediterranean. Records indicate prior identifications of *B. melitensis* biovar 2 in Saudi Arabia, Iran and Türkiye.[32,33]

Notably, our findings corroborated the presence of this variant in cattle. It appears that a proportion of 207 out of 420 cows with detectable culture-positive results were found to carry *B. melitensis* in their milk and lymph node. This indicated the presence of *B. melitensis* infection in small ruminants which might have been transmitted to dairy cattle. As a result, it is crucial to thoroughly assess the effectiveness of the Rev.1 vaccination program in small ruminants. One way to achieve this was by examining the antibody titers of vaccinated animals, which could provide insights into the extent of vaccination coverage and its efficacy. Recent research conducted in Mongolia proposed that a protective herd immunity could be attained by vaccinating around 60.00% of the small ruminant population.[21] Due to the discovery that 97 out of 158 dairy cattle farms with positive *Brucella* results carried an infection of *B. melitensis*, it is proposed that *B. melitensis* infections in small ruminants still play a significant role in cattle infections. To verify this issue, the isolation of *B. melitensis* strains from sheep and goats is necessary for genetic contrast. The potential transmission of the infection to other cattle and humans could arise from cows expelling *B. melitensis*. Two isolations of *B. aborts* RB51 from two bovine lymph node confirmed that they received RB51 dosage. However, it proposes relying solely on RB51 vaccination proves inadequate for managing brucellosis in areas where the disease is prevalent. To effectively address this widespread issue, it is essential to integrate vaccination with comprehensive control measures. These measures encompass implementing livestock management practices among farmers, initiating educational initiatives to enforce stringent hygiene protocols and undertaking the removal of seropositive dairy cattle. By combining these approaches, awareness about this pervasive ailment among farmers can be heightened, potentially leading to a reduction in the prevalence of brucellosis.[5,34,35]

In conclusion, the outcomes of our study, employing classical and molecular techniques, revealed the manifestation of brucellosis infection in dairy cattle farms of Iran despite of vaccination. This manifestation indicated the proliferation of diverse biovars of *B. melitensis* and *B. abortus*. Geographical distribution revealed that *B. melitensis* biovar 1 was the most common in dairy cow farms that this issue highlighted the complexity of bovine brucellosis in Iran and illustrated that *B. melitensis* was spread from small ruminants to cattle.

Serological methods unquestionably play a crucial role in identifying and detecting brucellosis in domesticated Bovidae. The techniques of RBPT, SAT, and I-ELISA are frequently employed to assess animal sera in initiatives aimed at managing livestock disease. The RBPT, functioning as rapid and cost-effective point-of-care assessments, carry the risk of producing inaccurate positive outcomes due to their potential to react with various non-*Brucella* antigens. This particular method has found widespread application as a rapid test, offering greater sensitivity albeit at the expense of reduced specificity. A few comprehensive investigations regarding the circulating *Brucella* isolates in dairy cattle farms of Iran are available. Nevertheless, despite this recent information, there are significant gaps that still exist in Iranian literature about how prevalent *B. melitensis* is in cattle. It is absolutely necessary to conduct more extensive research in order to fully understand the disease spread in dairy cattle farms. This understanding will eventually serve as the foundation for creating potential strategies to manage and stop the spread of bovine brucellosis in the endemic area. The Iranian government has recognized the severity of the situation and has made efforts to combat bovine brucellosis through vaccination campaigns, educational programs and improved diagnostic methods. However, these measures have been met with limited success due to financial constraints and the vast geographical spread of the disease.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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