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Brucella melitensis in Iranian Dairy Cattle: Isolation, Molecular Identification, and Biotyping

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ABSTRACT

Background: Brucellosis, caused by *Brucella* species, remains a significant zoonotic and economic challenge in Iran, where *Brucella melitensis* is increasingly detected in dairy cattle due to close contact with infected sheep and goats. The persistence of *B. melitensis* highlights the limitations of existing control strategies and the need for improved approaches. Investigating *B. melitensis* in Iranian dairy cows is crucial due to their interaction with mixed sheep and goat populations.

Methods: In 2021, 73 lymphoid tissue samples were collected from reactor-positive cows in seven Iranian provinces. Samples were analyzed using traditional bacteriological methods, biotyping, and molecular tools such as AMOS-PCR and Bruce-ladder PCR for strain identification and characterization. Samples were collected from seven provinces in Iran, namely Tehran, Kerman, Isfahan, Alborz, Qazvin, West Azerbaijan, and Fars.

Results: Out of 73 lymph node samples collected from seven Iranian provinces, 15 (20.55%) tested positive for *B. melitensis* through culture and PCR. Culture results confirmed uniform growth conditions with no significant differences among provinces. Molecular analysis validated the identity of all isolates, with AMOS-PCR amplifying a 731 bp product and Bruce-ladder PCR confirming field strains through species-specific DNA fragments. Biotyping revealed that 14 isolates were *B. melitensis* Biotype 1, distributed across six provinces, while one isolate from Kerman Province was Biotype 3.

Conclusion: The prevalence of *B. melitensis* in Iranian dairy cattle highlights significant gaps in existing vaccination programs and the urgent need for cross-species protective vaccines. Given its zoonotic risks and public health impact, enhanced surveillance, advanced diagnostics, and region-specific strategies are critical for effective control.The RB51 vaccine has notable limitations, including its inability to protect against *B. melitensis*, lack of standardized protocols, and failure to aid in brucellosis eradication. Collaboration among public health authorities, veterinarians, and policymakers is critical to address these challenges and mitigate the spread of *B. melitensis* in Iran.

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Introduction

Brucella species are Gram-negative, facultative intracellular coccobacilli that infect a wide range of hosts, including wildlife, domestic livestock, and humans (1, 2). Brucellosis, caused by *Brucella* spp., is a zoonotic disease of significant public health and economic importance, particularly in countries such as Iran. The disease primarily affects the reproductive system in livestock, leading to reduced productivity due to abortion, infertility, and decreased milk production (3). In humans, brucellosis manifests as a febrile illness with the potential for chronic complications and severe health outcomes (2). Transmission occurs primarily through consuming unpasteurized dairy products, contact with infected animals, and, less commonly, blood transfusion (4) or exposure to contaminated materials (5, 6). The incidence of the disease in Iran has recently risen from 21 to 25 cases per 100,000 people, highlighting the growing urgency of addressing this issue (7).

Brucellosis control in Iran has a long history; brucellosis has been recognized as a significant health challenge in Iran for decades, with the first major discoveries and control measures dating back to the 1920s (8). In 1932, *Brucella melitensis* was first isolated from human blood culture by Dr. Crandall, then head of the Pasteur Institute of Iran. Later, in 1948, *B. melitensis* was isolated from goat milk by Dr. Entesar at the Brucellosis Department of the Razi Vaccine and Serum Research Institute (RVSRI) (9), marking an important milestone in the understanding of the disease's zoonotic potential.

Efforts to combat brucellosis in livestock began in 1949 when the RVSRI introduced the S19 vaccine, initially targeting dairy farms in Iran. This vaccine was widely used until the end of 2006 and played a crucial role in disease management (8). However, the S19 vaccine had a significant drawback: it interfered with standard serological diagnostic tests, complicating the differentiation between vaccine-induced immunity and natural infection with field strains. To address this issue, the *B. abortus* RB51 vaccine, derived from strain 2308 and licensed in the United States in 1996, was introduced in Iran in 2002. By 2007 in Iran, the RB51 vaccine had completely replaced the S19 vaccine, as it did not produce interfering antibodies, thus resolving diagnostic challenges (1, 10, 11). This historical progression reflects Iran's long-standing efforts to combat brucellosis through scientific research, vaccine development, and strategic disease control measures.

In Iran, *B. melitensis* and *Brucella abortus* are the most common species responsible for brucellosis in livestock, with *B. abortus* predominantly infecting cattle and *B. melitensis* introduced via close contact with infected sheep and goats (12). Although regulatory control programs, including vaccination and culling, have been in place for decades, the persistence of brucellosis in livestock populations reflects the limitations of current strategies, particularly the lack of effective cross-species protection in vaccines like the RB51 (13, 14). Furthermore, B. melitensis poses an additional challenge as it often remains clinically undetectable in cattle (9, 12). Notably, *B. melitensis* infections in cattle can complicate the serological detection of *B. abortus* infections (15).

Globally, effective brucellosis control programs have successfully eradicated the disease in some countries, such as Japan, Canada, and New Zealand. However, in endemic regions like Iran, the disease remains widespread due to mixed-species grazing systems, nomadic husbandry practices, and inadequate veterinary infrastructure (9, 11, 13, 16). Understanding *Brucella*'s circulating strains and biovars is critical for designing effective control strategies. Among the known *Brucella* species, *B. melitensis* Biovar 1 is recognized as the most pathogenic and invasive for humans and livestock in Iran (17). However, limited knowledge exists about the molecular epidemiology and strain diversity of *B. melitensis* in dairy cattle populations.

Research on brucellosis is critical for understanding its complex epidemiology, improving diagnostic methods, and developing effective control strategies, as it remains a globally significant zoonotic disease causing substantial economic losses in livestock and severe public health risks, particularly in endemic regions (1, 18). Given the adaptability of *Brucella* to new environments and the emergence of novel epidemiological patterns, this study aims to identify the circulating strains of *B. melitensis* in dairy cattle across multiple provinces in Iran. The findings of this research will provide critical insights into the epidemiology of brucellosis in the country, contributing to the development of targeted control and eradication programs to safeguard animal and public health.

Materials and Methods

Sample Collection

This study was conducted in 2021 and involved sampling lymphoid tissues from reactor-positive cows, identified based on their serum titers of 1:160 or above, using the Rose Bengal and standard tube agglutination tests. According to the Iran brucellosis eradication program and test-and-slaughter (T/S) strategies in the cattle population, animals tested positive in the serological assays were transported to slaughterhouses, where they were slaughtered and collected. All the cows had a history of receiving a full dose of the strain RB51 vaccine (Iriba strain) at four to twelve months of age and were raised in closed systems within industrial farms. The cows were transported to slaughterhouses in seven provinces of Iran: Tehran, Kerman, Isfahan, Alborz, Qazvin, West Azerbaijan, and Fars.

A total of 73 lymphoid tissue samples were collected from reactor-positive animals during post-mortem inspection. The lymph nodes sampled included the retropharyngeal, mediastinal, supramammary, iliac, and prescapular (superficial cervical) lymph nodes. Sampling was carried out under aseptic conditions using sterile instruments to minimize the risk of contamination.

Each sample was carefully labelled with information on the cow's identification, sampling site, and location of the slaughterhouse. The collected tissues were immediately placed into sterile, leak-proof containers and kept at 4°C during transportation to the Brucellosis Department of the RVSRI for further analysis. Standard operating procedures for handling, processing, and analyzing the samples were followed, including adherence to biosafety guidelines for handling potentially infectious materials.

Bacteriological Examination

For bacteriological examination, a portion of each lymph node sample was inoculated onto Farrell's medium. The medium was prepared using a *Brucella* Agar Medium Base (Oxoid, CM0169) supplemented with a *Brucella* Selective Supplements (Oxoid, SR0083A), which contained Polymixin B (2,500 IU), Bacitracin (12,500 IU), Cycloheximide (50.0 mg), Nalidixic Acid (2.5 mg), Nystatin (50,000 IU), and Vancomycin (10.0 mg) per 500 mL of medium. Additionally, the medium was enriched with 5% sterile inactivated horse serum (Oxoid, SR0035C) and 2% of filter-sterilized solution of dextrose. Duplicate cultures were prepared for each sample and incubated at 37 °C under aerobic and microaerobic conditions (Micro-aerobic kit, Merck, Anaerocult C) for 7–14 days. Cultures were inspected daily for the growth of colonies.

Suspected *Brucella* colonies were identified based on colony morphology and growth characteristics, followed by confirmatory biochemical and phenotypic tests. These tests included Gram staining, modified Ziehl–Neelsen (MZN) staining, catalase, oxidase, and urease activity. All bacteriological procedures followed the established guidelines of the World

Organisation for Animal Health (WOAH) and employed classical identification methods to ensure accurate detection of *B. melitensis* (6, 19-23).

Biotyping

Classical biotyping was conducted following the methodology outlined by Alton et al. The analysis utilized *Brucella* monospecific antisera (A and M) and the *Brucella* reference phage of Tbilisi (Tb) (19), both of which were routinely prepared and employed at the RVSRI for diagnostic and analytical purposes.

A comprehensive panel of biotyping tests was performed, including evaluating CO_2 requirements and hydrogen sulfide (H_2S) production. Additional tests included assessing bacterial growth in the presence of thionine and basic fuchsin, agglutination by acriflavine, and lysis by specific bacteriophages. The results were interpreted based on established criteria and documented reference data to confirm the biotype of the isolates.

Genomic DNA Extraction

Genomic DNA was extracted from the isolated *Brucella* bacteria using a High-Purity PCR Template Preparation Kit (Roche, Germany), following the manufacturer's protocol. The integrity of the extracted DNA was verified by electrophoresis on a 1% agarose gel, and DNA concentration was measured at 260/280 nm using a Nanodrop Spectrophotometer (Wilmington, DE, USA). Extracted DNA samples were stored at −20 °C until further analysis.

Molecular Analysis

Molecular identification of the bacterial isolates was performed using IS711-based polymerase chain reaction (AMOS-PCR) and Bruce-ladder PCR to confirm the presence of *Brucella* species.

AMOS-PCR was carried out using five primers targeting the IS711 gene (Table 1). The PCR thermal cycling conditions were as follows: an initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 60 seconds, and extension at 72 °C for 3 minutes. The final extension was performed at 72 °C for 10 minutes. The reaction mixture had a total volume of 25 µL, containing 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.5 mM of each primer, 10 mM Tris–HCl (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.05 IU of Taq polymerase (24). Molecular typing was conducted using Bruce-ladder multiplex PCR, targeting eight specific *Brucella* genes (Table 1). The thermal cycling conditions included an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 56 °C for 90 seconds, extension at 72°C for 3 minutes, and a final extension at 72 °C for 10 minutes. The PCR reaction components were identical to those used in AMOS-PCR. B. melitensis reference strain 16M was used to confirm the amplification of the 731 bp product characteristic of *B. melitensis*. *B. abortus* reference strain 544 was included to verify species differentiation and ensure no cross-reactivity with *B. abortus*. A reaction mixture without template DNA was used as the negative control to detect any potential contamination or nonspecific amplification (25-27).

The amplified AMOS-PCR and Bruce-ladder PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. The gels were visualized under UV illumination to determine the presence and size of specific DNA fragments corresponding to the expected amplicons.

Statistical analysis

The data were entered into a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. The data obtained were

analyzed using IBM SPSS Statistics, version 25.0. Analysis of Variance (ANOVA) was employed to evaluate variations within the data. A significance level of $p < 0.05$ was used to determine statistical significance.

Results

Isolation and identification

Out of the 73 lymph node samples collected from seven provinces in Iran, a total of 15 (10.95%) samples tested positive for *B. melitensis* through both culture and PCR, with the majority of isolates identified as Biotype 1 and a single isolate classified as Biotype 3. In Qazvin, 30 lymph nodes were sampled, and five tested positive for B. melitensis using both culture and PCR. Fars Province had 11 samples, with three positive cases, while Isfahan had five samples, of which two tested positive. In Tehran, 10 lymph nodes were sampled, and one tested positive. West Azerbaijan also showed two positive cases out of 11 sampled lymph nodes (Figure 1).

Bacteriological

Out of the 73 lymph node samples collected from seven provinces in Iran, 15 (20.55%) samples were confirmed as *B. melitensis* through bacteriological culture and subsequent biochemical analyses. The *B. melitensis* isolates exhibited growth on selective culture media within a typical incubation period of 3 to 7 days, consistent with established guidelines for cultivating *Brucella* species. No significant variation in growth time was observed across the positive samples, suggesting uniformity in the culture conditions and isolation process ($p>0.05$). The ANOVA results indicate no statistically significant differences in the mean growth times among the provinces ($p > 0.05$) (Table 2).

Colonies exhibited small glossy honey colonies with smooth surfaces and translucent appearances typical of *B. melitensis*. Gram staining revealed Gram-negative coccobacilli. The isolates were somewhat acid-fast. Biochemical testing provided further confirmation (Table 3). All isolates displayed characteristic reactions, including catalase and oxidase positivity, urease activity, and distinct growth patterns under specific conditions. These results on the side resistance to thionine and basic fuchsin dyes, no required CO_2 for growth, and did not produce H_2S enabled the accurate identification and differentiation of *B. melitensis* from other *Brucella* species.

Biotyping

Biotyping was performed on all positive *B. melitensis* isolates to determine their biovars. Of the 15 (20.55%) positive cases, 14 (19.7%) isolates were identified as Biotype 1. The remaining isolate (1.40%) from Kerman Province was identified as Biotype 3 (Figure 1).

Molecular Analysis

Molecular confirmation of *B. melitensis* was conducted using AMOS-PCR and Bruce-ladder PCR assays. All 15 isolates produced a specific PCR product of 731 bp in the AMOS-PCR assay, confirming their identity as *B. melitensis* (Figure 2). This amplification is characteristic of the IS711 insertion sequence unique to this genus and species. The results in the Bruce-ladder PCR assay further validated the isolates as *B. melitensis*. All isolates demonstrated amplification of specific PCR products with sizes of 152 bp, 450 bp, 587 bp, 794 bp, 1,071 bp, and 1,682 bp, which are hallmark features of the field strain of *B. melitensis*. The molecular analysis results corroborate the bacteriological and biochemical findings, establishing a comprehensive confirmation of *B. melitensis* in the positive samples.

Table 1. Primer sequences, target genes, and expected amplicon sizes for Bruce-ladder PCR were used to identify and differentiate *B. melitensis* and related *Brucella* species.

Primer*	Primer Sequence (5'-3')	Target gene	Amplicon size (bp)	Ref.
IS711-BM	TGCCGATCACTTTCAAGGGCCTTCAT AAATCGCGTCCTTGCTGGTCTGA	IS711	731	(1)
IS711-AB	TGCCGATCACTTTCAAGGGCCTTCAT GACGAACGGAATTTTTCCAATCCC	IS711	498	(1)
IS711-ovis	TGCCGATCACTTTCAAGGGCCTTCAT CGGGTTCTGGCACCATCGTCG	IS711	976	(1)
IS711-suis	TGCCGATCACTTTCAAGGGCCTTCAT GCGCGGTTTTCTGAAGGTTCAGG	IS711	285	(1)
BMEI0998f BMEI0997r	ATC CTA TTG CCC CGATAA GG GCT TCG CAT TTT CACTGT AGC	Glycosyltransferase, gene *wboA*	1,682	(2)
BMEI1436f BMEI1435r	ACG CAG ACG ACC TTCGGTAT TTT ATC CAT CGC CCTGTCAC	Polysaccharide deacetylase	794	(2)
BMEII0428f BMEII0428r	GCC GCT ATT ATG TGGACT GG AAT GAC TTC ACG GTCGTT CG	Erythritol catabolism, gene *eryC* (d-erythrulose-1-phosphate dehydrogenase)	587	(2)
BMEI0535f BMEI0536r	GCG CAT TCT TCG GTTATG AA CGC AGG CGA AAA CAGCTA TAA	Immunodominant antigen, gene *bp26*	450	(2)
BMEII0987f BMEII0987r	CGC AGA CAG TGA CCATCA AA GTA TTC AGC CCC CGTTAC CT	Transcriptional regulator, CRP family	152	(2)
BMEII0843f BMEII0844r	TTT ACA CAG GCA ATCCAG CA GCG TCC AGT TGT TGTTGA TG	Outer membrane protein, gene *omp31*	1071	(2)
BR0953f BR0953r	GGA ACA CTA CGC CACCTT GT GAT GGA GCA AAC GCTGAA G	ABC transporter binding protein	272	(2)
BMEI0752f BMEI0752r	CAG GCA AAC CCT CAG AAG C GAT GTG GTA ACG CAC ACC AA	Ribosomal protein S12, gene *rpsL*	218	(2)

* The designations are derived from the *B. melitensis* (BME) or *B. suis* (BR) genome sequences, with "f" indicating forward primers and "r" indicating reverse primers. - The *B. melitensis* Rev.1 vaccine strain is distinguished from other *B. melitensis* strains by the presence of a unique 218-bp fragment. The 272-bp fragment is unique to B. suis, *B. canis*, and *B. neotomae* (27).

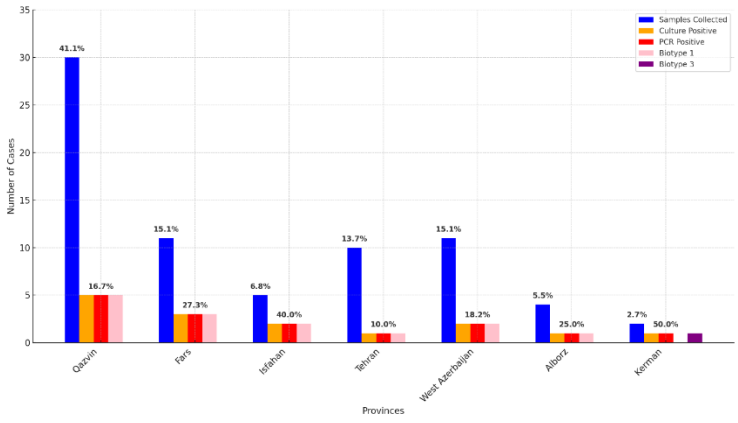

Fig 2. Distribution of *B. melitensis* Cases and Biotypes by Province. The percentages for the blue column were calculated from the total number of samples, which is 73.

Table 2. Incubation times for the growth of *B. melitensis* isolates across different provinces.

Province	Number of Positive Samples	Mean Growth Time (Days)	Standard Deviation (Days)
Qazvin	5	4.8	0.45
Fars	3	4.9	0.35
Isfahan	2	4.7	0.50
Tehran	1	5.0	-
West Azerbaijan	2	4.8	0.40
Kerman	1	5.0	-
Alborz	1	4.9	-

Table 3. Biochemical Results for *B. melitensis* Isolates (23).

Biochemical Test (*B. melitensis*)	Result
Gram Staining	Gram-negative
Colony Morphology	Small, smooth, translucent
Catalase Test	Positive
Oxidase Test	Positive
Urease Test	Positive
H_2S Production	Negative
CO_2 Requirement	Negative
Growth in Thionine (Under 40 µg/ml)	Resistant
Growth in Basic Fuchsin	Resistant
Agglutination with Antisera A	Only biotypes 3
Agglutination with Antisera M	Biotypes 1 and 3
Sensitivity to Tbilisi Phage	Negative

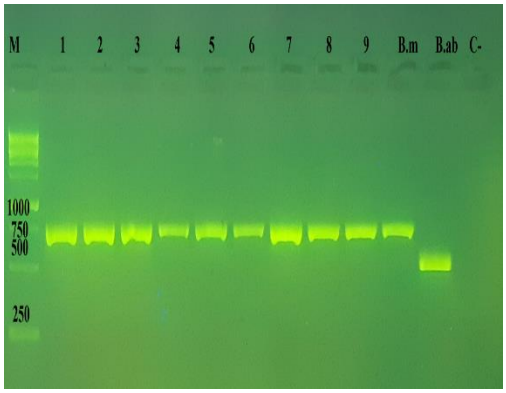

Fig 2. Gel electrophoresis results of AMOS-PCR assay for *B. melitensis* isolates. Lanes 1–9 represent the tested isolates, all showing amplification of a specific 731 bp product characteristic of *B. melitensis*. Lane M indicates the DNA ladder (molecular size marker), lane B.m serves as the positive control (*B. melitensis* reference strain 16M), lane B.ab represents the *B. abortus* control, and lane C- is the negative control with no amplification. The consistent amplification across lanes confirms the identity of the isolates as *B. melitensis*.

Discussion

The findings of the present study provide significant insights into the epidemiological distribution of *B. melitensis* in Iran, highlighting its predominance among dairy cattle. The findings suggest that *B. melitensis* infection in cattle remains a remarkable issue for both animal and public health in Iran. While *B. abortus* is the primary cause of brucellosis in Iranian dairy cows (12, 28), understanding the presence of *B. melitensis* biovars in cattle herds is crucial due to its heterogeneous nature and greater challenges in eradication. The significance of studies like the current investigation is underscored by the high risk *B. melitensis* poses to humans, as it is the most dangerous *Brucella* species and requires only a minimal inoculum to cause infection (2, 29). The detection of *B. melitensis* Biotype 1 as the most common strain across the provinces, along with the identification of Biotype 3 in Kerman Province, aligns with previous reports on the geographic prevalence and strain diversity of *Brucella* species in the region (1, 3, 13, 16, 17, 30). These results underline the importance of tailored strategies for controlling and eradicating brucellosis in Iran, where endemic conditions pose unique challenges.

The predominance of *B. melitensis* Biotype 1 in dairy cattle across the sampled provinces reflects its capacity to adapt to cattle as a secondary host (31). This adaptability is facilitated by the pathogen's ability to invade and persist in phagocytic and non-phagocytic host cells, aided by virulence factors such as lipopolysaccharide and the Type IV secretion system (32). The detection of Biotype 3 exclusively in Kerman may indicate localized risk factors, including the possible introduction of infected animals or cross-species transmission from nearby small ruminant flocks. The geographic location of Iran, bordered by countries with limited veterinary infrastructure, such as Pakistan, Iraq, and Afghanistan, exacerbates the risk of brucellosis spread through animal trafficking and porous borders (33, 34). Weak border quarantine systems and the nomadic nature of livestock husbandry further complicate control efforts (11).

Interestingly, the present study revealed the isolation of *B. melitensis* from cattle raised in intensive industrial farms. This finding challenges the assumption that *B. melitensis* transmission is limited to cattle in close contact with sheep and goats, as observed in studies from southern Europe and western Asia (12, 35). The present study underscores the potential role of forage as a source of *B. melitensis* contamination. Forage sourced from agricultural lands where sheep and goats graze throughout the year could harbor *B. melitensis*, making its presence in industrial farm settings plausible. Supporting this observation, a study by Khamesipour et al. detected *B. melitensis* in 4 out of 102 (3.92%) semen samples collected from cattle and buffaloes in 43 industrial farms producing semen. These findings highlight the critical need for monitoring forage sources and their role in the transmission dynamics of *B. melitensis* in industrial livestock systems. They also emphasize that intensive cattle production systems in Iran face remarkable risks from sheep and goats, necessitating increased vigilance and preventive measures.

The current investigation successfully employed a combination of classical and molecular techniques, including AMOS-PCR and Bruce-ladder PCR, to confirm the presence of *B. melitensis*. The consistency between the results of the phenotypic methods and DNA-based tools reinforces the reliability of these diagnostic approaches. As noted by Dal et al., ópez-Goñi et al., and Wareth et al., multiplex PCR is a rapid and accurate method for *Brucella* species identification and biovar differentiation, offering advantages over traditional culture and serological tests (27, 36, 37). The study by Demirpence et al., like the present study, utilized traditional culture techniques in combination with molecular methods to isolate *B. melitensis* from tissue samples and confirm its presence in livestock populations (38). The present study also demonstrates the uniformity of isolation conditions, with no significant differences in

growth times across provinces, validating the robustness of the culture methods used. While the combination of these techniques has proven effective in confirming *B. melitensis*, it is important to consider the potential for false negatives or misidentification, particularly in mixed infections or low-bacterial-load scenarios. This highlights the need for continuous refinement of diagnostic methods in veterinary microbiology (5).

The present study's findings align with and expand upon the conclusions drawn in the meta-epidemiological study by Dadar et al. While Dadar et al. identified *B. melitensis* as the most prevalent species in Iranian livestock, the current study highlights explicitly the predominance of *B. melitensis* Biotype 1 in dairy cattle across seven provinces. This biovar-specific analysis provides additional granularity to the epidemiological data, offering insights into the regional distribution of *Brucella* strains. Both studies emphasize the utility of PCR-based diagnostic tools for *Brucella* detection, with our results confirming the reliability of Bruce-ladder PCR in differentiating *Brucella* species and biovars (3).

The findings of the present study align with those of Zowghi et al., particularly in highlighting the predominance of *B. melitensis* Biovar 1 as the most prevalent biovar in Iran. Zowghi et al. reported *B. melitensis* Biovar 1 in the majority of their isolates (2102 cases), spanning multiple host species, including sheep, goats, cattle, and humans. Similarly, the current study emphasizes the widespread presence of *B. melitensis* Biovar 1 in dairy cattle across several provinces, with Biovar 3 localized exclusively in Kerman Province. This geographic specificity for Biovar 3 is consistent with Zowghi et al.'s findings, where it represented a smaller proportion (106 cases) of isolates, reflecting its limited distribution compared to Biovar 1 (13). The findings of the present study align with those of Erami et al. in confirming *B. melitensis* Biovar 1 as the dominant biovar responsible for brucellosis. While Erami et al. focused on human cases in Kashan, Iran, and identified Biovar 1 exclusively among isolates, the present study expands this understanding to dairy cattle across multiple provinces, further emphasizing the widespread prevalence of this biovar. Both studies highlight the importance of biotyping in understanding the epidemiological distribution of *Brucella* species (39).

The study by Sharifiyazdi et al. highlights the low prevalence of *B. melitensis* infection in milk samples of dairy cows in Iran, focusing on the presence of Biovar 1 and Biovar 2 in specific provinces. Their study emphasizes the mixed population dynamics of sheep, goats, and cattle as a source of *B. melitensis* introduction into dairy herds. They underscores the potential risks posed by *B. melitensis* as a nonspecific agent in cattle herds, reflecting its role as a zoonotic pathogen with public health implications. Combining traditional and molecular diagnostic methods in both studies underscores their importance in providing reliable epidemiological data for effective brucellosis management. Similar to the present study, the mentioned study employed traditional biotyping methods to classify the isolates (12).

The present study's findings align with regional and international observations of *B. melitensis* biovars in cattle. For instance, *B. melitensis* Biovar 3 has been reported in cattle in several countries, underscoring its emerging significance in non-preferred hosts. Buyukcangaz and Sen documented the first isolation of *B. melitensis* Biovar 3 from a bovine aborted fetus in Turkey, a country employing the *B. abortus* S19 vaccine to eradicate brucellosis (35), while Di Giannatale et al. found that 36.4% of *Brucella* strains isolated from cattle in Italy between 2001 and 2006 were *B. melitensis* Biovar 3. Their study also identified other strains, such as *B. abortus* biovars 1, 3, and 6, as prevalent in cattle in Italy. Di Giannatale emphasized that managing *B. melitensis* infections in cattle is especially difficult, as the *B. abortus* vaccines used in Italy do not provide effective

protection against *B. melitensis* (29). These reports, combined with the results of the present study, emphasize the importance of monitoring and controlling *B. melitensis* Biovar 3 in cattle, given its zoonotic potential and ability to adapt to diverse host systems (4).

Brucellosis control in Iran faces numerous challenges, including insufficient economic compensation for culling infected animals, weak enforcement of animal health laws, and the limited efficacy of existing vaccines in cattle. The RB51 vaccine, while effective in controlling *B. abortus*, lacks cross-species protection for *B. melitensis* (1, 3, 11, 16). An ideal vaccine for cattle must provide effective cross-species protection, yet current vaccines fall short. While *B. abortus* vaccines, such as RB51, are widely used, they offer limited efficacy against *B. melitensis*. Similarly, the *B. melitensis* Rev.1 vaccine has not been fully evaluated for cattle use, leaving a critical gap in protection strategies (29). The Iranian Veterinary Organization has implemented a control program involving RB51 vaccination alongside test, slaughter, and quarantine measures. However, there is insufficient data on RB51's efficacy in preventing *B. melitensis* infection in cattle herds (12, 28, 40). These limitations highlight the need for further research into the immune response to B. melitensis in cattle and the development of vaccines that can address both *B. abortus* and *B. melitensis*. For effective control, strategies must combine targeted vaccination with robust epidemiological monitoring and tailored eradication programs to address the unique challenges posed by *B. melitensis* in mixed-host systems (2, 12, 22, 41).

The findings of the present study contribute to the growing body of evidence highlighting the limitations of the RB51 vaccine in controlling brucellosis. Despite its widespread adoption in some countries, no country has successfully eradicated brucellosis using RB51, and in several cases, the vaccine has been abandoned after prolonged use (42). Interestingly, in 1996, the United States, the country that introduced RB51, had nearly eradicated the disease without relying on the RB51 vaccine (43). One of the most critical shortcomings of RB51 is its inability to provide immunity against *B. melitensis*, as was evident in this study, where *B. melitensis* was isolated from vaccinated cattle (42, 44). Moreover, three decades after its introduction, there is still no standardized operating procedure for RB51, and its effectiveness and safety under various physiological and epidemiological conditions remain unproven (45). This lack of consistency undermines confidence in the vaccine and highlights the need for alternative strategies. The RB51 vaccine is not an ideal choice for controlling brucellosis, as its perceived efficacy is often based on the misconception that "protection against abortion" is a sufficient measure of success (46). This approach overlooks the critical issue that RB51-vaccinated but infected cows can continue to shed virulent *Brucella* in vaginal fluids and milk, perpetuating the disease within herds. Moreover, a proportion of calves born to these cows, although seronegative, are congenitally infected and pose significant risks once they reach sexual maturity, further undermining the long-term effectiveness of the vaccine (42, 45).

The ideal brucellosis vaccine, as discussed in prior studies, should provide robust, long-lasting protection with a single dose while being safe, stable, and easy to differentiate from field strains. While the RB51 vaccine was initially introduced to address some of these requirements, it falls short of critical benchmarks, particularly in its inability to prevent infections caused by *B. melitensis*. Furthermore, RB51 does not address the fundamental challenges of mass vaccination in endemic regions, including adverse effects in pregnant cattle and diagnostic confusion in differentiating vaccinated animals from infected ones. These deficiencies underscore the need to revisit more effective alternatives, such as the S19 vaccine, which has demonstrated greater efficacy in eradication programs when combined with T/S

strategies. Programs integrating S19 vaccination with T/S strategies have proven effective in eradicating cattle brucellosis in regions where the disease has been successfully controlled (42, 45). This study reinforces the urgency of developing vaccines that can address the limitations of current options and provide comprehensive protection against *Brucella* species in diverse epidemiological contexts.

Controlling *B. melitensis* infection in cattle presents significant challenges, particularly in regions like Iran, where *B. abortus* and *B. melitensis* coexist. As a key component of the national milk industry, dairy cattle can act as reservoirs, posing a risk of contaminated milk being distributed widely (12). Furthermore, the control strategies for *B. abortus* and *B. melitensis* differ, requiring tailored approaches for each pathogen. In contrast to *B. abortus*, the scarcity of studies and reports on *B. melitensis* infection in cattle has made its occurrence less apparent and drawn less attention to this issue. The elimination of bovine brucellosis in developing countries like Iran is hindered by financial constraints, limiting compensation for farmers whose animals are culled (1, 2, 12, 47, 48).

The prevalence of *B. melitensis* in dairy cattle underscores its zoonotic significance as the primary causative agent of human brucellosis in Iran. Given its potential to infect both humans and animals, effective surveillance and control measures are critical (9, 49). Comprehensive strategies should include enhanced diagnostic capabilities to detect latent infections, strict animal movement controls, and education campaigns targeting farmers to improve cooperation with veterinary services. Successful eradication programs in countries like Switzerland, Germany, and the Netherlands demonstrate that a combination of rigorous testing, vaccination, and economic compensation is essential for long-term control (2, 14, 50, 51).

Iran must adopt a multi-faceted approach involving veterinarians, sociologists, and economists to combat brucellosis effectively. This topic includes designing affordable and sustainable control plans that incentivize farmer cooperation, enforcing stricter border controls to prevent the introduction of infected animals, and improving the quality and organization of veterinary services. International collaboration with neighboring countries is also critical to address the regional nature of the disease (1, 4, 9).

Conclusion

The present investigation findings underscore the need for targeted control measures, improved surveillance systems, and vaccination programs that offer cross-species protection against *B. melitensis*. Special attention should be paid to addressing the gaps in existing vaccination programs, including the limitations of the RB51 vaccine, and exploring novel vaccines that provide broad protection against Brucella species. Public health authorities and veterinary services should work collaboratively to strengthen brucellosis control programs, incorporating strategies such as mass vaccination of livestock, regulated animal movement, and strict border controls to prevent cross-border transmission.

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Ethics approval and consent to participate

This study was conducted in compliance with ethical guidelines for animal research. Written consent was obtained from the owners of the animals prior to sampling. All samples were collected using standard sampling methods.

Conflict of interest

The authors declare that they have no conflict of interest.

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