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Research article

Shedding rate of Brucella spp. in the milk of seropositive and seronegative dairy cattle

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ABSTRACT

Brucellosis in cattle herds has caused severe economic losses in many regions worldwide. A cross-sectional study was performed to investigate the presence of *Brucella* spp. in industrial dairy cattle farms in Iran. For this purpose, 935 blood and 935 milk samples were randomly collected from industrial dairy cattle farms in Iran's Alborz and Tehran provinces. Blood and milk samples were collected on the same day from each cow. Serological, bacteriological, and molecular characterization of *Brucella* isolates were performed using standard methods. Our results revealed the seroprevalence of brucellosis in dairy cattle farms in the Alborz and Tehran provinces, reaching 19.8%, 6.7%, 5.1%, 14.1%, and 13.1% using the Rose Bengal plate test (RBPT), serum agglutination test (SAT), 2-mercaptoethanol test (2-ME), indirect enzyme-linked immunosorbent assay (i-ELISA) and milk ring test (MRT), respectively. Furthermore, the results of bacterial culture and PCR analyses showed the presence of *Brucella abortus* among dairy cattle in the Alborz province and *Brucella melitensis* and *B. abortus* among dairy cattle in the Tehran province. Moreover, statistical analysis with Cohen's Kappa has highlighted the near-perfect agreement between RBPT and i-ELISA (k = 0.86). In contrast, substantial agreement was shown between RBPT and SAT performance (k = 0.70) and moderate agreement between RBPT and 2-ME (k = 0.67). The findings of this investigation showed shedding of *Brucella* in the milk of seropositive cows, which is a serious problem involving the maintenance and further spread of *Brucella* infection on the farm. Therefore, for brucellosis detection or eradication in dairy cattle farms, bacteriological and serological tests of milk samples should be performed along with blood analysis to inhibit the uncontrolled spread of the disease in animals and humans.

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1. Introduction

Different species of the genus *Brucella* cause brucellosis as a contagious zoonotic disease that mainly infects domestic animals, such as cattle, buffaloes, camels, goats, and sheep. Bovine brucellosis remains a persistent problem in middle and low-income countries [1,2]. In Iran, bovine brucellosis caused by *B. abortus* and *B. melitensis* is an endemic disease [3,4]. Most infected animals shed Brucellae through milk, vaginal secretions, urine, amniotic fluid, fetal membranes, and semen. Bacteria invade the gravid uterus, fetus, and placenta and induce abortion in the second half of gestation [5]. Although these infections are often lifelong, most abortions in infected cows occur only once, and these animals remain an infection source until the next calving [6]. Therefore, the reproductive performance in carrier animals may remain unaffected, making it difficult to control the disease [7,8]. Bovine brucellosis may also lead to severe economic losses through the extra costs of breeding improvements, increased calving intervals, death of the young stock, stillbirth, and abortion [9]. Despite some vaccine-associated reproductive issues [3,10], RB51 live attenuated vaccine has decreased the prevalence of *B. abortus* infections, playing a pivotal role in eradication programs and improving the control of clinical signs in contaminated herds [6,11]. Several reports have demonstrated that seronegative animals could hamper the influential role of the control program and might spread the brucellosis to susceptible hosts and facilitate spillover into the environment [12–15]. Generally, *Brucella* spp. infection of raw milk can occur through exogenous infections after or during milking by environmental factors or through endogenous infections from the blood of contaminated livestock [16,17]. The incidence of *Brucella* spp. in contaminated sources in the dairy farm environment provides critical information for high-risk populations, suggesting the role of *B. melitensis* and *B. abortus* in human brucellosis through the consumption of cattle, goats, sheep, and camel milk [17]. Standard diagnostic laboratory tests for *Brucella* identification used in Iran include RBPT, SAT, MRT, and i-ELISA, followed by Polymerase Chain Reaction (PCR) [18]. However, *Brucella* isolation remains the gold standard for definitive brucellosis diagnosis and is not readily available in all parts of the country. A meta-analysis in Iran revealed an overall prevalence of 14.7% for cattle as the most affected livestock by brucellosis [18]. However, some investigations have shown a high seroprevalence of up to 18.5% in Iranian cattle [18]. There are few studies on bovine brucellosis, particularly in Iran's western and northern regions. Considering these reports regarding the high seroprevalence of brucellosis in dairy cattle farms, this study aimed to investigate the occurrence of *Brucella* spp. in dairy cattle herds to estimate the presence of bacteria in raw milk and serum samples.

2. Material and methods

2.1. Ethical considerations

Ethical approval for the study was granted by the Research Ethics Committee, Iranian veterinary organization, Tehran, Iran (Approval Code: IR. IVO.1398.006). All animals in this study were treated according to the ethical standards for field studies approved by the Iranian veterinary organization Tehran, Iran. The dairy farmers were informed about the purpose of the investigation and gave their informed consent.

2.2. Sampling and study area

This cross-sectional descriptive study was performed from December 2017 to July 2019 in intensive dairy cattle farms in the Alborz and Tehran provinces (western and northern Iran). A sampling of random farms was performed in a country's industrial area with high milk production volumes. This study analyzed sera and milk samples obtained from 935 dairy cows from 31 intensive dairy farms. A total of 21 intensive farms in Alborz province and 10 in Tehran province were randomly selected. The farming system crowded large groups of cows in confined indoor spaces. Approximately 10 ml of blood drawn from the jugular vein was collected from 675 to 260 dairy cattle from Alborz province and Tehran province, respectively, with more than one year of age. Blood samples were preserved without anticoagulant in a cold box and immediately transported to the Veterinary Diagnosis and Treatment Department (Karaj, Iran). Individual milk specimens (675 samples from Alborz province and 260 samples from Tehran province) were also obtained from all bled cows. Blood and milk samples were collected on the same day from each cow. After serological analysis using the Rose Bengal plate test (RBPT), serum agglutination test (SAT), 2-mercaptoethanol test (2-ME), and indirect enzyme-linked immunosorbent assay (i-ELISA) on blood sera, we separated the cows into seropositive and seronegative dairy cattle. A sample was regarded as seropositive for *Brucella* when it tested positive in both agglutination tests, such as RBPT, SAT, and 2-ME, and non-agglutination tests, such as i-ELISA.

2.3. Serological tests

Serum samples (n = 935) were collected and tested using the Rose Bengal plate test (RBPT), serum agglutination test (SAT), and 2-mercaptoethanol test (2-ME). The *Brucella* antigen was produced at the Razi Vaccine and Serum Research Institute (Karaj, Iran). According to the OIE standard guidelines, a titer of 1:80 or greater for SAT and 2 ME was considered positive for specific agglutination *Brucella* antibodies [19]. Furthermore, an indirect enzyme-linked immunosorbent assay (i-ELISA) was performed to confirm the positive results in dairy cattle. I-ELISA was performed using an Ingezim *Brucella* Bovina 2.0 ELISA test kit (Ingenasa, Madrid, Spain) following the manufacturer's instructions (https://ingenasa.eurofins-technologies.com). This kit was specifically intended to evaluate specific antibodies against *Brucella* antibodies. The OD values of the tested samples were recorded at 450 nm, and positive samples were detected according to the following formula:

Cut off = OD_{450} nm positive Control × 0.4 = 40% positivity. The OD of samples was considered positive for O.D. value ≥ 1.0 and negative for OD value ≤ 0.2.

Milk samples obtained from dairy cattle were kept at 4 °C overnight prior to testing using the milk ring test (MRT). Then, one drop of MRT antigen was mixed with 1 ml of milk sample in a narrow tube, gently mixed, and kept for 1 h at 37 °C. If the antibody binds to the antigen and forms a blue ring above the white milk column, the *Brucella* antibody is present in the milk. Antibodies were absent if the mixture remained homogeneously bluish-white throughout the tube [20].

2.4. Bacteriological examination of milk samples

Milk samples (675 samples from Alborz province and 260 samples from Tehran province) of all dairy cattle were cultured on *Brucella*-selective supplement media containing nalidixic acid (2.5 mg), polymyxin B sulfate (2,500 IU), vancomycin (10 mg), nystatin (50,000 IU), cycloheximide (50 mg), and bacitracin (12,500 IU) (HiMedia, Mumbai, India). They were then maintained at 10% CO_2 at 37 °C for 14 days [21].

2.5. Molecular typing

According to the manufacturer's protocol, DNA was extracted from the isolated bacteria using the Exgene Cell SV kit (Gene All, South Korea). DNA integrity was checked on a 1% agarose gel. Furthermore, DNA concentration was evaluated at 260/280 nm using an ND-1000 Nanodrop (Wilmington, DE, USA) [22]. The IS*711*-based PCR for *Brucella* identification was performed on the extracted DNA by AMOS (*abortus melitensis ovis suis*)-polymerase chain reaction (Table 1) under the following PCR conditions: initial denaturation at 95 °C for 5 min (Step 1), denaturation at 95 °C for 30 s (Step 2), annealing at 55 °C for 60 s (Step 3), extension at 72 °C for 3 min (Step 4) and final extension at 72 °C for 10 min (Step 5). Steps 2, 3, and 4 were repeated 35 times [23]. Molecular typing by multiplex PCR (Bruce-ladder) with 16-primer multiplex PCR was performed to differentiate the wild-type of *Brucella* spp. and S19, RB51, and Rev1 vaccines. The Bruce-ladder PCR process was as follows: denaturation at 95 °C (5 min), 35 cycles of denaturation at 95 °C (30 s), annealing at 56 °C (90 s), extension at 72 °C (3 min) and a final extension at 72 °C (10 min) [24]. The PCR products were separated by 1.5% agarose gel electrophoresis. All the PCR primers used are listed in Table 1.

2.6. Data analysis

Data analyses were performed by SPSS software version 22. For each serological test, the number of positive samples for brucellosis was divided by the total number of dairy cattle, and the prevalence of brucellosis was determined. A paired chi-square (x2) analysis was performed by McNemar to evaluate the results of different serological tests in dairy cattle. Differential incidence was considered significant when the *p*-value was less than 0.05. The degree of agreement between various serological methods, i.e., RBPT, SAT, 2-ME, and i-ELISA, was evaluated using Cohen's kappa coefficient (κ) based on their overall agreement in the brucellosis diagnosis tests. The

Table 1
Specific primer sets and expected amplicon sizes for different *Brucella* species.

Strain amplicon	Primer set	Primer sequence (5-3′)	DNA target	size (bp)	References
AMOS PCR	IS711	TGCCGATCACTTTCAAGGGCCTTCAT	alpha-ketoglutarate dependent dioxygenase	498	[23]
	AB	GACGAACGGAATTTTTCCAATCCC			
AMOS PCR	IS711	TGCCGATCACTTTCAAGGGCCTTCAT	hypothetical protein	731	[23]
	BM	AAATCGCGTCCTTGCTGGTCTGA			
AMOS PCR	IS711	TGCCGATCACTTTCAAGGGCCTTCAT	TRAP transporter solute receptor, TAXI	976	[23]
	B.ovis	CGGGTTCTGGCACCATCGTCG	family protein		
AMOS PCR	IS711	TGCCGATCACTTTCAAGGGCCTTCAT	indole-3-glycerol phosphate synthase	285	[23]
	B.suis	GCGCGGTTTTCTGAAGGTTCAGG			
Bruce-ladder PCR	BMEI0998f	ATC CTA TTG CCC CGATAA GG	Glycosyltransferase, gene wboA	1,682	[24]
	BMEI0997r	GCT TCG CAT TTT CACTGT AGC			
Bruce-ladder PCR	BMEI0535f	GCG CAT TCT TCG GTTATG AA	Immunodominant antigen, gene bp26	450	[24]
	BMEI0536r	CGC AGG CGA AAA CAGCTA TAA			
Bruce-ladder PCR	BMEII0843f	TTT ACA CAG GCA ATCCAG CA	Outer membrane protein, gene omp31	1071	[24]
	BMEII0844r	GCG TCC AGT TGT TGTTGA TG			
Bruce-ladder PCR	BMEI1436f	ACG CAG ACG ACC TTCGGTAT	Polysaccharide deacetylase	794	[24]
	BMEI1435r	TTT ATC CAT CGC CCTGTCAC			
Bruce-ladder PCR	BMEII0428f	GCC GCT ATT ATG TGGACT GG	Erythritol catabolism, gene eryC	587	[24]
	BMEII0428r	AAT GAC TTC ACG GTCGTT CG	(Derythrulose-1-phosphate dehydrogenase)		
Bruce-ladder PCR	BR0953f	GGA ACA CTA CGC CACCTT GT	ABC transporter binding protein	272	[24]
	BR0953r	GAT GGA GCA AAC GCTGAA G			
Bruce-ladder PCR	BMEI0752f	CAG GCA AAC CCT CAG AAG C	Ribosomal protein S12, gene *rpsL*	218	[24]
	BMEI0752r	GAT GTG GTA ACG CAC ACC AA			
Bruce-ladder PCR	BMEII0987f	CGC AGA CAG TGA CCATCA AA	Transcriptional regulator, CRP family	152	[24]
	BMEII0987r	GTA TTC AGC CCC CGTTAC CT			

perfect agreement was considered if Kappa was 0.81–1.00, strong agreement for 0.61–0.80, moderate agreement for 0.41–0.60, fair agreement for 0.21–0.40, and slight agreement for 0.01–0.20 [25].

3. Results

3.1. Serological analysis

Using RBPT, the first serological screening was performed in all dairy cattle. One hundred thirty-three samples (19.7%) from seven dairy cattle farms (33.4%) in Alborz province were RBPT-positive. For further confirmation, all serum samples were subjected to SAT, 2-ME, and i-ELISA. The results of the SAT and 2-ME tests showed a significantly lower percentage of seropositivity than RBPT, with 5.8% (39/675) and 3.4% (23/675) of positive cases, respectively ($p < 0.05$) (Table 2). In contrast, the i-ELISA appeared relatively more specific, with 13.5% (91/675) positive results (Table 2). A sample was regarded as seropositive for *Brucella* when it tested positive in both agglutination tests, such as RBPT, SAT, and 2-ME, and non-agglutination tests, such as i-ELISA. In addition, the milk sample from each cow in the Alborz province was subjected to MRT for the specific detection of *Brucella* antibodies in all animals. According to MRT, 12.4% of the milk samples were positive. MRT was positive in 60.9% (81/133) of seropositive cows and 0.5% (3/542) of seronegative cows. Fifteen farms (71.5%) had at least one positive animal by i-ELISA. Moreover, serological analysis from Tehran province revealed that 53 samples (20.3%) distributed among four dairy cattle farms were RBPT-positive. In contrast, 9.2%, 9.6%, and 15.7% of the sera showed positive reactions in the SAT, 2-ME, and i-ELISA tests, respectively. According to the MRT, 14.6% of the milk samples in Tehran province were positive, and all positive MRT results were in seropositive cows.

3.2. Bacteriological isolation

Nine hundred thirty-five milk samples from dairy cattle were subjected to bacterial examination on selective *Brucella* agar. *Brucella* spp. were recovered and identified in 8.6% (57/675) of milk specimens collected from seropositive cows in Alborz province and 6.5% (17/260) of milk specimens collected from seropositive cows in Tehran province. The isolated bacteria showed a common specific phenotypic characteristic of *Brucella* spp. All bacterial isolates were grown at 37 °C after a 5-day incubation with 10% carbon dioxide (CO2). *Brucella* was visible under the microscope as small pairs or single Gram-negative coccobacilli that showed small, round, and pin-point colonies with translucent and shiny honey-colored surfaces (Fig. 1). All 57 bacteria isolated from the milk samples in Alborz province and 17 from dairy cattle in Tehran province were seropositive in RBPT, SAT, 2-ME, i-ELISA, and MRT.

3.3. Molecular confirmation

AMOS PCR tests on isolated bacteria led to the characterization of bacteria isolated from the milk of seropositive dairy cattle in the Alborz and Tehran provinces (Table 2). *The B. melitensis* gene was detected in 11 isolated bacteria from Tehran province using the AMOS PCR method. The *B. abortus* gene was detected in 63 bacteria isolated from milk samples of the Tehran and Alborz provinces. In the current study, *B. melitensis* resulted in a PCR product and a specific band of 731 bp in AMOS PCR, allowing the detection of biovars 1, 2, and 3, whereas *B. abortus* resulted in a PCR product with a specific band of 498 bp, allowing the detection of biovars 1, 2, and 4 (Fig. 2). Bruce-ladder analysis also identified the isolated bacteria as wild-type *B. melitensis* by PCR products of 1682, 794, 587, 450, 152, and 1,071bp in size and wild-type *B. abortus* with five fragments of 1,682, 794, 587, 450, and 152 bp in size (Fig. 3). However, through Bruce-ladder analysis, none of the isolated bacteria were recognized as vaccine strains in this study.

3.4. Comparative analysis for Brucella ssp. antibodies detection

Our study showed that the rate of brucellosis detection was remarkably higher in RBPT analysis than in other serological tests, including SAT, 2-ME, and i-ELISA ($p < 0.05$). Cohen's kappa coefficient used an inter-rater reliability analysis to evaluate the agreement within different serological methods. A near-perfect agreement was found between RBPT and i-ELISA ($k = 0.86$, perfect agreement), whereas a substantial agreement was observed in the comparison of RBPT and SAT ($k = 0.70$), as well as a moderate

Table 2
Results from the Rose Bengal plate test (RBPT), serum agglutination test (SAT), 2-mercaptoethanol test (2-ME), indirect enzyme-linked immunosorbent assay (i-ELISA), Milk ring test (MRT), culture and PCR on the blood and milk samples of dairy cattle farms.

Location	Number of cattle	RBPT N (%) positive	SAT N (%) positive	2-ME N (%) positive	iELISA N (%) positive	MRT N (%) positive	Milk Culture N (%) positive	PCR	Species (N)
Alborz	675	133 (19.7)	39 (5.8)	23 (3.4)	91 (13.5)	84 (12.4)	57 (8.6)	57 (8.6)	*B. abortus* (57)
Tehran	260	53 (20.3)	24 (9.2)	25 (9.6)	41 (15.7)	38 (14.6)	17 (6.5)	17 (6.5)	*B.melitensis* (11) *B. abortus* (6)
Totals	935	186 (19.8)	63 (6.7)	48 (5.1)	132 (14.1)	122 (13.1)	74 (7.9)	74 (7.9)	*B. melitensis* (11) *B. abortus* (63)

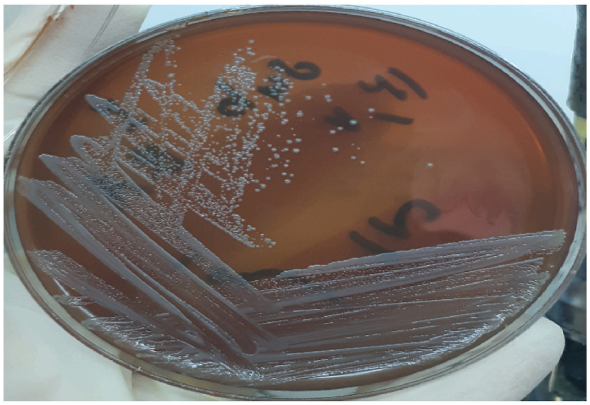

Fig. 1. Colony of isolated *B. melitensis* on blood agar.

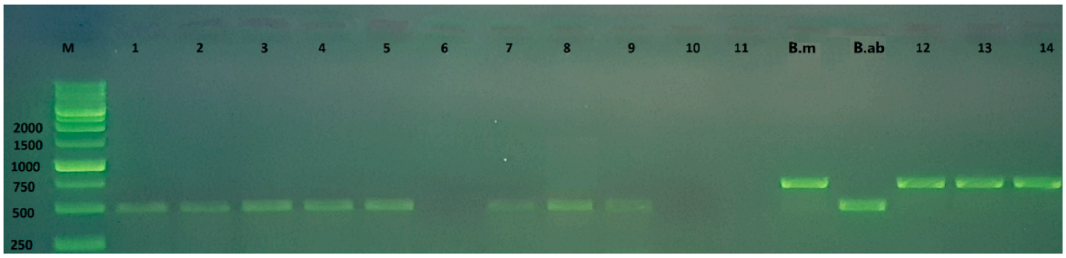

Fig. 2. Agarose gel (1%) electrophoresis of PCR amplified products generated from DNA samples in AMOS-PCR. M, molecular marker 1000 base pairs (bp), columns 1, 2, 3, 4, 5, 7, 8, and 9: band 498 bp of *Brucella abortus* bacteria isolated from the milk samples; column 6 and 10 negative bacteria in AMOS PCR; column 11: negative control; B. ab: *Brucella abortus* reference strain; B.m: *Brucella melitensis* reference strain, column 12–14: *B. melitensis* bacteria isolated from the milk samples showing the band 731bp.

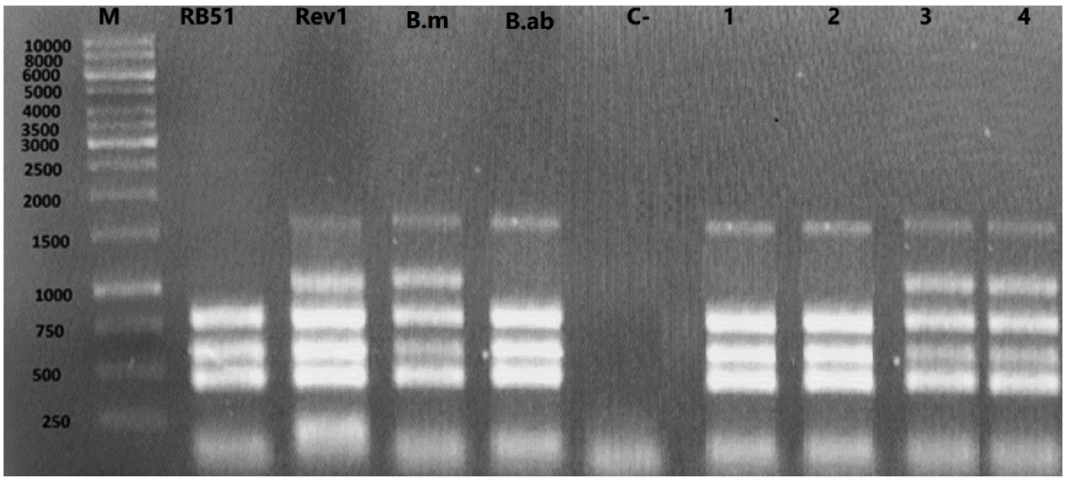

Fig. 3. Agarose gel (1%) electrophoresis of PCR amplified products generated from DNA samples in Bruce-ladder PCR. Lane M shows a DNA size marker (1000bp DNA ladder). Lane RB51 shows *B. abortus* RB51 vaccine strain; Lane Rev1: *B. melitensis* Rev1 vaccine strain; Lane B.m: *B. melitensis* reference strain; Lane B. ab: *B. abortus* reference strain; Lane 6 negative control, Lane1 and 2: B. *abortus* field strains; Lane 3 and 4: B. *melitensis* field strains.

agreement between RBPT and 2-ME (k = 0.67).

4. Discussion

Brucellosis is one of the most important zoonotic diseases prevailing in the large and small ruminants of the Middle East, including Iran [10,16,26]. Vaccination is vital for preventing and eradicating infections and is the most economical control component [8]. However, the administration of currently available vaccines alone is insufficient to eliminate brucellosis in any host species, including cows. Control programs for brucellosis usually lead to failure or, at best, restricted decreases in disease incidence or the prevalence of human brucellosis [27]. Therefore, new brucellosis vaccines with high safety and efficacy are in high demand and can be more widely applied under field conditions to address the diversity of host species. Brucellosis in Iranian dairy cattle is predominantly associated with *B. abortus* biovar 3 in large ruminants, the most prevalent biovar [4]. The prevalence of bovine brucellosis is a critical hazard to milk consumers in both urban and rural populations [28]. The seroprevalence of brucellosis in dairy cattle was evaluated in the center of Iran using the RBPT, SAT, and i-ELISA. The overall seroprevalence at the individual levels was 5.6%, 3.9%, and 4.9%, respectively [29]. In contrast, our results showed a significantly higher prevalence among serum samples from dairy cattle in western Iran. Another study from northern Iran showed that the prevalence rate of bovine brucellosis in cattle breeds was 4.72% [30]. The difference in *Brucella* prevalence in certain geographic areas of Iran could be influenced by some risk factors, including animal species, age, pregnancy conditions, dairy production procedures, hygienic conditions, animal husbandry practices, herd size, breeding approaches, and socioeconomic status [31–33].

In Iran, vaccination with the RB51 strain is practiced in the bovines of dairy herds. In this study, we chose a battery of conventional serological tests, i.e., RBPT, SAT, i-ELISA, 2-ME, and MRT, to test the occurrence of brucellosis by field strains in dairy cattle. Serology remains vital in the diagnosis of brucellosis in animals and humans. These serological tests are suitable for screening animals in disease-endemic areas. However, the isolation of *Brucella* remains the gold standard diagnostic criterion but requires advanced biosafety and biosecurity levels (BSL), for example, BSL-3. The isolated bacteria must be confirmed and characterized based on biochemical and molecular biological criteria. Hence, we chose serology and isolation for confirmatory diagnosis of brucellosis in dairy cattle farms in an endemic area.

In the present study, serological screening by RBPT led to the highest rate of *Brucella* seropositivity, reaching 19.8% of the 935 tested samples. Not surprisingly, screening by RBPT resulted in a higher proportion of positive cases compared to other serological tests such as SAT, 2-ME, and i-ELISA. RBPT is reported to have a lower specificity than 2-ME and may thus reveal false positives in animals [34–36]. Intriguingly, our results showed two samples with seronegative responses in the RBPT and positive responses in the i-ELISA. These false-negative results in the RBPT test could be due to the prozone effect and the high antibody content in the sera [37]. Our study also reported the isolation of *B. abortus* and *B. melitensis* from milk samples of seropositive cows maintained in intensive dairy farms. Several studies have already reported the occurrence of *B. abortus* and *B. melitensis* infections in vaccinated cattle [3,12,38,39]. Some factors have been designated as responsible for these infections, such as improper vaccination methods and schedules, the replacement or upgrading of cattle through the purchase of infected animals from the market, breaches in biosecurity, management systems, demographic factors, wildlife interaction, age, breed, sex, lactation status, and climate [40–44]. *Brucella* seropositivity in vaccinated cattle may lead to severe diagnostic difficulties and concerns regarding the efficacy of government control programs for disease prevention [12,38]. Moreover, *Brucella*-infected cows may remain undiagnosed, constituting a hidden disease transmission source. Our study showed that i-ELISA is more accurate than RBPT and correlated better with culture and MRT experiments (Table 2). These results agree with those obtained by several investigations reporting a better specificity for an i-ELISA diagnostic method for screening brucellosis [45,46]. Therefore, although the isolation of *Brucella* spp. by culture is the gold standard for brucellosis diagnosis, it presents several restrictions in control programs, such as the need for optimal sampling and storage conditions of biological specimens, highly skilled personnel, and high-security laboratory facilities. Moreover, due to extended time for culture results and hazardous procedures, serological approaches are often given preference for the first screening and detection of *Brucella*-specific antibodies in livestock [35].

However, concerning i-ELISA having high specificity and sensitivity, seropositive cows should be evaluated for infection by other field strains associated with a cross-reaction caused by bacterial LPS, including *Salmonella* sp., *Escherichia coli*, *Escherichia hermannii*, *Yersinia enterocolitica,* and *Vibrio cholerae* [47,48]. Furthermore, cows in endemic areas such as Iran have constant contact with field strains of *Brucella* and could therefore develop a memory against this antigen through an immunological response [48]. The results of our study demonstrated the isolation of field strains of *B. abortus* and *B. melitensis* from dairy cows using PCR. Cows contaminated with *B. abortus* and *B. melitensis* in their milk samples had been vaccinated as adults with full and reduced RB51 doses. The isolation of *Brucella* species from milk samples of cows has been reported previously [14,49]. The incidence of *Brucella* antibodies in the milk of seronegative animals was also reported in our study. This might be because of the small number of *Brucella* spp. in the bloodstream that cannot produce antibodies to induce a humoral response resulting in seronegative reactions. Several studies have also reported the presence of *Brucella* species in seronegative cows in endemic areas [12–14]. However, MRT was introduced as a more specific test for brucellosis detection than the RBPT, SAT, CFT, and ELISA methods and can replace them [50]. However, the use of this test is restricted by the quality of milk [35]. The false-positive results of MRT could be revealed at the end of the lactation period in milk with colostrum and in animals with mastitis [51].

Vaccination and preventive measures, such as biosecurity and quarantine, are needed to reduce the occurrence of brucellosis at these farms. Routine screening of animals is necessary, even after vaccination. Culture examinations should complement routine serological testing. Vaccinated herds and milk from these cows should be handled using protective measures to reduce zoonotic transmission. RBPT is an excellent serological screening tool in low-income countries such as Iran. Finally, developing more efficacious

and safer vaccines alone, combined with increased emphasis on other regulatory program measures, could significantly reduce the worldwide prevalence of brucellosis and associated zoonotic infections. Control measurements utilized in dairy cattle farms and accurate screening tests by serological and bacterial analyses are vital for the control program's removal of all positive animals. The pasteurization of milk before human consumption has been highly proposed. However, the possible limitations of this study are the relatively small number of farms analyzed and the need for more conventional biotyping for *Brucella* identification. Further studies could extend the sample load and monitoring period and focus on other differentiation and identification methods for *Brucella* spp. to be quicker and less expensive. Molecular typing is an option that has already been investigated.

Author contribution statement

Anahita Emadi, and Shojaat Dashtipour: Conceived and designed the experiments.
Faranak Abnaroodheleh: Performed the experiments, Wrote the paper.
Tariq Jamil: Analyzed and interpreted the data, Wrote the paper.
Amin Mousavi Khaneghah and Maryam Dadar: Contributed reagents, materials, analysis tools or data, Wrote the paper.

Data availability statement

No data was used for the research described in the article.

Declaration of interest's statement

The authors declare no competing interests.

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