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Research Paper

Detection of *Brucella* Spp. in Raw Milk and Dairy Products of Traditional Domestic Dairy Sale Centers by Real-time PCR in Semnan

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ABSTRACT

Introduction: Brucellosis, also known as Malta fever, is a significant zoonotic infectious disease in Iran, causing considerable public health and economic complications. Human infection is primarily acquired through the consumption of unpasteurized milk and dairy products. Conventional diagnostic approaches, such as culture and serology, are limited by low sensitivity, biosafety risks, and potential cross-reactivity. Molecular methods, particularly real-time polymerase chain reaction (real-time PCR), offer greater sensitivity and specificity. This cross-sectional study, investigated the presence of *Brucella* spp. in raw milk and traditional dairy products (cream, cheese, and ice cream) collected from local markets in Semnan, Iran.

Materials & Methods: A total of 95 samples were analyzed using real-time PCR with SYBR Green dye, targeting the *bcsp31* gene for genus-level detection and species-specific primers for *Brucella abortus* and *Brucella melitensis*. Additionally, the limit of detection (LOD) was evaluated using the serial dilution of *Brucella* standard strain.

Results: The results revealed that among the samples tested, four samples were positive for *Brucella* spp. Specifically, three samples were confirmed as *B. abortus*, and one sample was identified as *B. melitensis*. Following propagation, DNA fragments of 498 bp and 731 bp, corresponding to *B. abortus* and *B. melitensis*, respectively, were successfully detected in the infected samples. Moreover, no significant overall difference in *Brucella* prevalence was found across products and bacterial load analysis revealed higher median colony-forming units (CFU) in cheese compared to milk and ice cream.

Conclusion: These findings highlight the presence of *Brucella* contamination in dairy products sold through informal channels, emphasizing the need for improved monitoring, strict control strategies, and consumer education to reduce disease transmission.

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1. Introduction

Brucellosis is among the most widespread zoonotic diseases in Iran, with profound public health and economic consequences [1]. In humans, the disease affects various tissues and organs and can be categorized as acute, sub-acute, or chronic forms, often leading to prolonged illness and disability [2]. *Brucella melitensis* and *Brucella abortus* are the most pathogenic species for humans and can infect a wide range of domestic animals, including, cattle, buffalo, goats, sheep, and camels. In animals, brucellosis is associated with abortions, stillbirth, weak calves, genital infections, placentitis, epididymitis, and orchitis, with bacteria excreted through uterine discharges and milk. Human infection occurs primarily via the consumption of raw or unpasteurized dairy products or through direct contact with infected animals. *Brucella* species can survive in raw milk for 10 days, in fresh cheese for up to 3 months, and in ice cream and cream for some time [3]. Foodborne diseases are an important public health problem as they not only affect human health, but also have a significant impact on economic and trade issues. Studies linking different pathogens in food to the disease in humans would help quantify the risk of foodborne diseases. Moreover, the prevention of foodborne diseases in general is a complex effort, involving many different actors along the chain of production from the farm to food service [4, 5]. Globally, the World Health Organization (WHO) estimates approximately 500000 new human cases annually.

Despite vaccination campaigns and control measures, brucellosis remains endemic in parts of Middle East, South America, and Asia. In Iran, surveys suggest that about 7.4% of cattle are infected. Transmission continues largely through the informal distribution of raw dairy products, particularly in rural communities with limited awareness of hygienic practices [6]. Conventional diagnostic methods, such as culture, are time-consuming, hazardous, and often yield false negatives due to the fastidious nature of the pathogen. Serologic methods, while rapid, may lack specificity because of cross-reactivity and insufficient antibody levels. Molecular techniques, particularly polymerase chain reaction (PCR) and real-time PCR (qPCR), provide superior sensitivity, specificity, and biosafety.

1.1. Real-time PCR [7, 8]

The use of qPCR has provided several advantages over conventional PCR, such as quantification, real-time, and in-situ analyses, in addition to automation. In this technique, the PCR products are detected as they accumulate, and the amount of generated PCR product is proportional to the increase in a fluorescent signal, which is monitored during the exponential phase. This technique the permits rapid identification and quantification of bacteria [9].

The present study aimed to investigate the presence of *Brucella* spp. in raw milk and traditional dairy products sold in Semnan, Iran. Given the social, economic, and geographical conditions of Semnan, where most people use traditional milk products, there is a possibility of people contracting this disease. Using real-time PCR with SYBR Green dye, we targeted genus-specific genes to detect *B. abortus* and *B. melitensis*. The findings provide insights into the prevalence of brucellosis in local dairy products and inform strategies for food safety and disease control.

2. Material and Methods

2.1. Samples preparation

A cross-sectional study involving three consecutive sampling rounds was conducted over approximately three months (early spring to late spring 2020) on traditional dairy products sold in Semnan Province, Iran. Based on a previously reported average prevalence of 6.6% for similar products, a 95% confidence level (Z=1.96), and a 5% margin of error, the sample size was calculated using Cochran's formula, yielding 95 samples [6]. The total number of samples was proportionally allocated to different dairy products according to their market sales volume, resulting in 50 raw milk samples, 23 traditional ice cream samples, 11 traditional cream samples, and 11 unpasteurized cheese samples, all collected randomly from traditional dairy sales centers.

2.2. DNA extraction

For each sample, 300 µL was processed using the Dyna Bio DNA Mini Kit (Takapouzist Co., Iran) according to the manufacturer's instructions. The purity and quantity of extracted DNA were measured using a NanoDrop spectrophotometer (Thermo Scientific Nanodrop, Wilmington, USA) at 260 and 280 nm (A260/280 ratio). Extracted DNA was eluted in 50 µL elution buffer. Primers targeted the *bcsp31* gene (223 bp fragment) for genus-level detection. Species-specific primers targeted a 498 bp fragment (*B. abortus*, *alkB* gene) and a 731 bp fragment (*B. melitensis*, *BMEI 1162* gene). The specifications of the primers used are listed in Table 1. The primers' specificity was assessed using BLAST [10].

2.3. Real-time PCR procedure

Amplification was performed using a Rotor-Gene (Q MDx, Germany). The amplification reactions contained 2 µL DNA template, 2.5 U AmpliTaq Gold DNA polymerase (Ampliqon, Copenhagen, Denmark), 1X (5 µL of 10X) GeneAmp buffer II, 6 mM $MgCl_2$, 800 µM GeneAmp dNTP blend (BioFact, Copenhagen, Denmark), 300 nM of each primer, and sterile water. The cycling conditions utilized in this experiment were as follows: an initial denaturation step was conducted at 95 ˚C for a duration of 10 minutes. This was succeeded by 40 cycles, which included denaturation at 95 ˚C for 10 seconds, primer annealing at 55 ˚C for 30 seconds, and extension at 72 ˚C for 30 seconds. Subsequently, a melting curve analysis was executed over a temperature range of 65 ˚C to 95 ˚C, with a transition rate of 0.1 ˚C/s, based on continuous fluorescence measurements. The positive control contained the Brucella strain ATCC 23456, while the negative control contained nuclease free water. PCR efficiency was evaluated by constructing a standard curve from serial dilutions of quantified Brucella control DNA. Cycle threshold (Ct) values were plotted versus log_{10} initial copy number. The slope of the regression line was used to calculate efficiency based on Equation 1:

1. *E=10−1/slope−1* [11, 12].

The limit of detection (LOD) was evaluated using the Brucella strain ATCC 23456, the standard strain was cultured in BHI broth overnight at 37 ˚C and then adjusted to a turbidity equal to 0.5 McFarland standard tube (0.08–0.1 absorbance at 600 nm wavelength). Then, eight standards (S1–S8) were prepared by 10-fold serial dilutions (from 1 to 0.00000001) of the adjusted BHI broth (equal to 0.5 McFarland). Genomic DNA was extracted from all standards, and quantitative real-time PCR was performed. The log linear phase of the reaction was used to determine the cycle threshold (Ct) for each standard. To obtain a reference line, the Ct values of the eight standards with known numbers of bacteria (S1=10^7; S2=10^6; S3=10^5; S4=10^4; S5=10^3; S6=10^2; S7=10; S8=1 bacteria per mL, respectively) were determined as previously described by Kralik and Ricchi. The linear regression equation obtained from the reference standard was employed to quantify *Brucella* spp. in genomic DNA, with the findings represented as log 10 CFU/g (colony-forming units per gram) [11, 12].

2.4. Statistical analysis

Differences in *Brucella* prevalence among dairy product types were assessed using chi-square and Fisher's exact tests. Bacterial load (CFU/g or mL) between products was compared using the nonparametric Kruskal–Walli's test, followed by the Mann–Whitney U test for pairwise comparisons. A $P<0.05$ was considered statistically significant.

3. Results

The real-time PCR technique was assessed through the analysis of the Ct value, melting temperature (Tm), specificity, sensitivity, and efficiency of the standard curve.

3.1. Molecular identification of *Brucella* spp. in milk and traditional dairy products using real-time PCR method

Among 95 samples analyzed using real-time PCR, four were positive for *Brucella* spp.; specifically, three contained *B. abortus*, and one sample in raw milk contained *B. melitensis* (Table 2). No significant overall difference in *Brucella* prevalence was found across products ($P=0.093$), and bacterial load analysis revealed higher median CFU in cheese (>10^7) compared to milk and ice cream ($P=0.067$).

3.2. Sensitivity and specificity of real-time PCR

To precisely pinpoint the target genes in *Brucella* spp. and determine the optimal annealing temperature for the primer sets in real-time PCR, we conducted a series of PCR reactions. The outcomes of these reactions were visualized through electrophoresis on a 1% agarose gel (Figure 1).

Table 1. Oligonucleotide primers used for the detection of *Brucella* spp. B. abortus and *B. melitensis* [10]

PCR Identification	Reverse Primer	Forward Primer	Fragment Length
Brucella spp	GGGTAAAGCGTCGCCAGAAG	GCTCGGTTGCCAATATCAATGC	223
B. melitensis	CATGCGCTATGATCTGGTTACG	GCGGCTTTTCTATCACGGTATTC	731
B. abortus	CATGCGCTATGATCTGGTTACG	AACAAGCGGCACCCCTAAAA	498

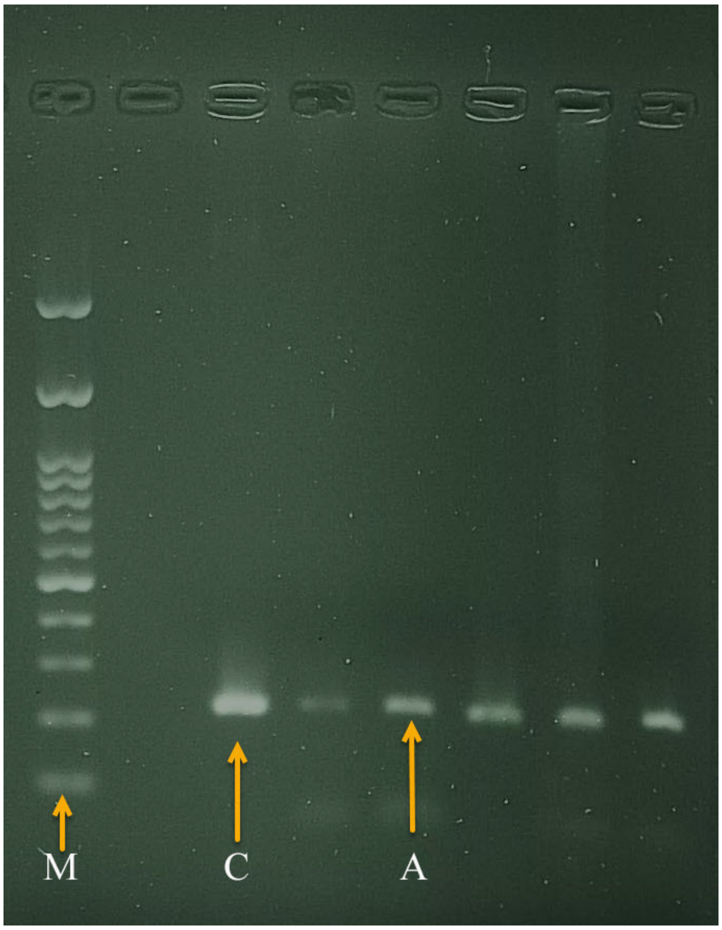

Figure 1. Agarose gel electrophoresis of PCR products generated from *Brucella* spp. at 55.3 °C.

Note: Lane M: Marker (100 bp ladder); Lane C: Positive control; Lane A: Amplified bcsp31 fragment (233 bp). Arrows indicate the expected amplicon bands.

The standard curve exhibited excellent linearity (R^2> 0.99) between $\log_{10}$ DNA concentration and Ct values. The slope was −3.26, corresponding to a reaction efficiency of 102%, which is well within the optimal range (90–110%) for qPCR assays (Figure 2).

The melting (Figure 3) and proliferation curves (Figure 4) for both positive samples and controls, utilizing targeted primers for the *bcsp31* gene, *alkB*, and *BMEI1162*, are presented to support the identification of the *Brucella* spp. and their relevant species (Figure 5). Results from the real-time PCR analysis demonstrated that three samples tested positive for *B. abortus* (*alkB* gene), while one sample was confirmed as *B. melitensis* (*BMEI1162* gene) among the four positive detections.

Table 2. The results of Real-Time PCR for distribution of *Brucella* spp. By product type

Type of product	No. of the samples	Frequency of PCR Positive Brucellosis	Percent of PCR Positive Brucellosis
Raw milk	50	1	2%
Traditional ice cream	23	1	4.34%
Cheese	11	2	18.18%
Traditional cream	11	-	-

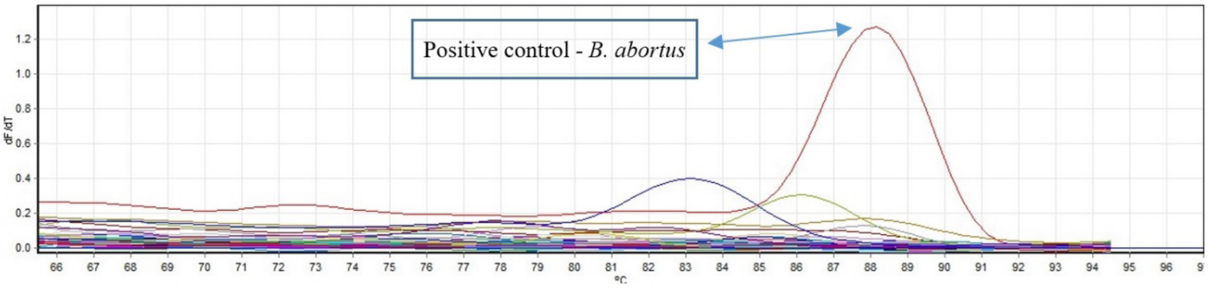

Figure 3. Melting peaks (left) and curves (right) of *Brucella* spp. positive samples in milk and dairy product samples, target Tm of about 86.5±2.5 °C (right). Melting peaks (left) and curves (right) of *Brucella* spp. positive samples in milk and dairy product samples, target Tm of about 86.5±2.5 °C (right)

3.3. Quantification of *Brucella* Spp. DNA concentrations in positive samples using a standard curve

The proliferation and melting curves for the dilutions derived from both the standard and positive samples are illustrated in Figure 6. It shows the drawn melting curve resulting from the reactions in the dilutions prepared from the positive control. These curves, which are all in the same temperature range, show the accuracy of the manufactured products and, as a result, the accuracy of the test.

3.4. The equation concentration of DNA in positive samples with the bacterial counts (CFU/gram or Cc)

A comparative analysis, performed utilizing a NanoDrop ND-1000 UV-Vis's spectrophotometer (Thermo Scientific NanoDrop, Wilmington, USA) as outlined in Table 3, reveals a significant correlation between CFU/mL and the yield of DNA obtained from standardized dilutions. This correlation is quantitatively represented by the equation derived from the standard curve depicted in Figure 7. Consequently, the quantification of DNA extracted from the positive samples was executed based on their positions along this standard curve, and the results are summarized in Table 4.

Standard curves demonstrated strong linearity between DNA concentration and bacterial counts (R^2 >0.99). The DNA concentration of positive samples ranged from 0.72 to 62.94 ng/ µL, corresponding to bacterial loads of 1-10^7 CFU/mL. Cheese rined samples showed the highest bacterial counts (>10^7 CFU/g).

4. Discussion

The detection of *Brucella* spp. in 4.21% of samples highlights the ongoing risk of brucellosis transmission through raw and traditional dairy products in Semnan, Iran. The higher prevalence in cheese (18.18%) compared with the milk and ice cream suggests that certain traditional products may provide favorite conditions for bacterial persistence.

Our findings align with previous studies in Iran, where contamination rates varied wildly depending on product type, region, and diagnostic methods. For examples, studies have reported contamination rates of 2-10% in

well within the optimal range (90–110%) for qPCR assays (Fig 2).

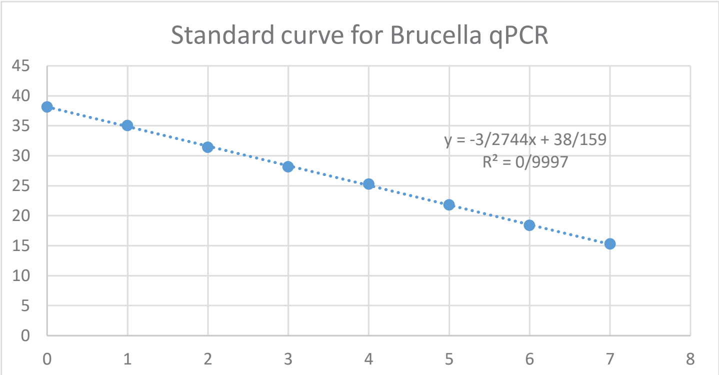

Figure 2. Standard curve for *Brucella* spp. qPCR assay (log_{10} copies vs Ct) slope=−3.27, R^2=0.9997, efficiency=102.02%

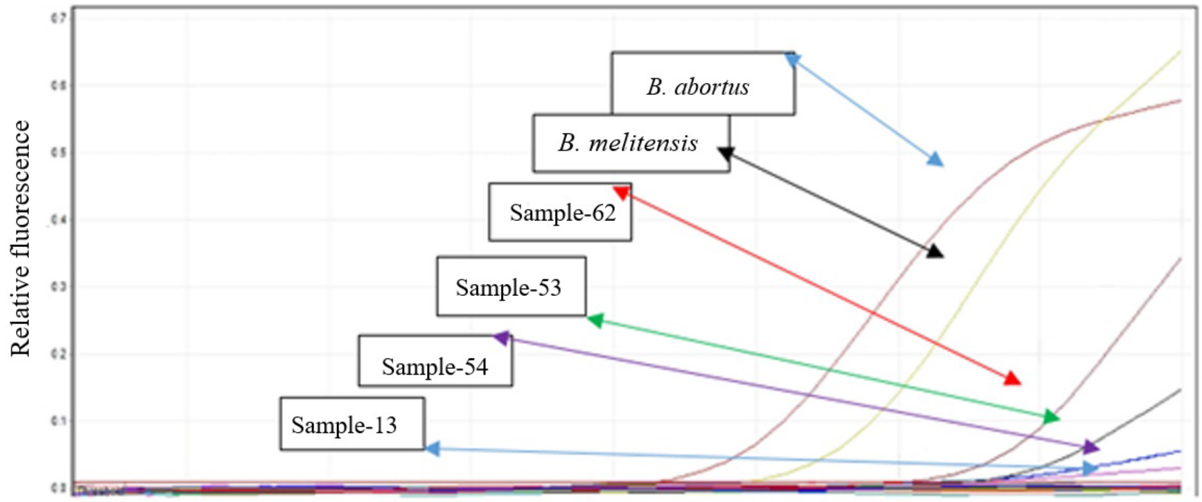

Figure 4. Proliferation curves: *B. abortus* (purple curve) and *B. melitensis* (yellow curve)

Note: *B. abortus*: Sample 62 (cheese rind) is shown by the brown curve, and sample 13 (cheese rind) is represented by the pink curve. *B. melitensis*: Sample 54 (traditional ice cream) is dark blue curve and sample 53 (raw milk) is gray curve

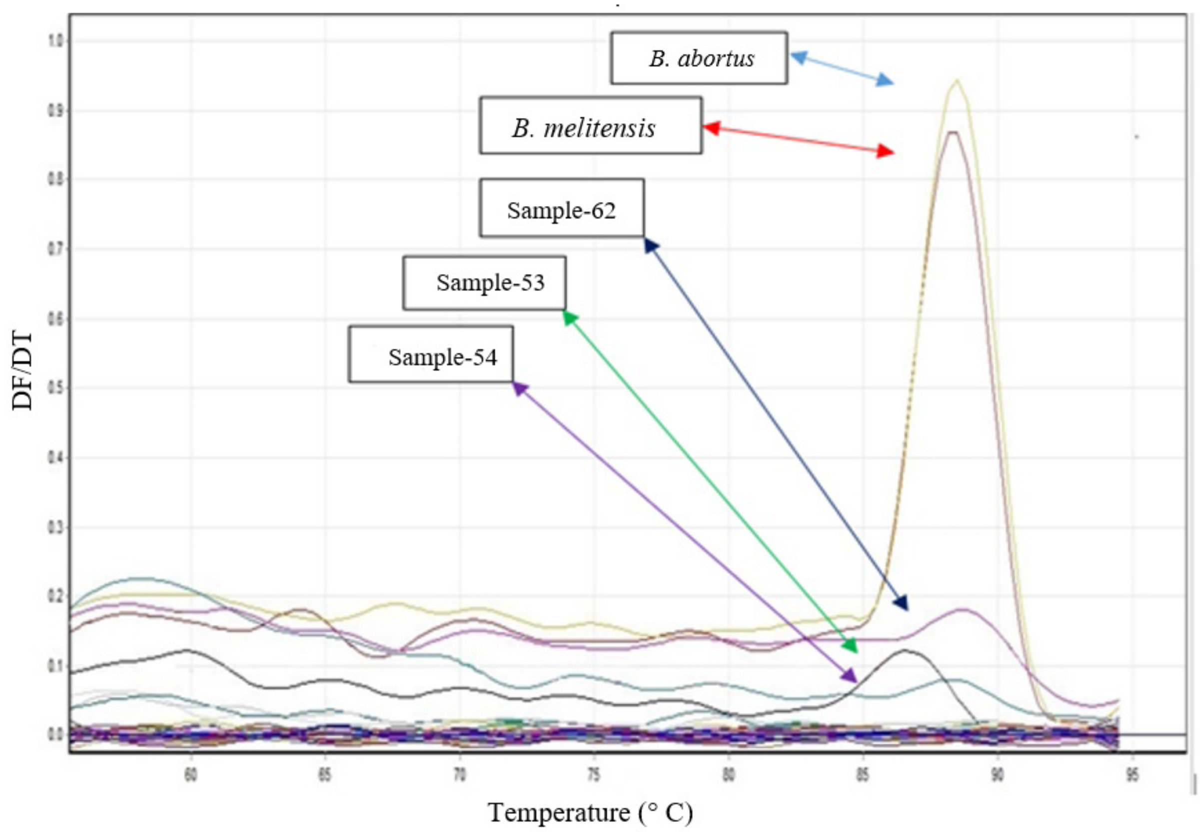

Figure 5. Melting curves of *Brucella* spp. identification for *BMEI1162* and *alkB* genes

Note: *B. abortus* (yellow curve): Sample 62 (cheese rind) is shown by the pink curve. *B. melitensis* (purple curve): Sample 54 (traditional ice cream) is gray curve, Sample 53 (raw milk) is blue curve.

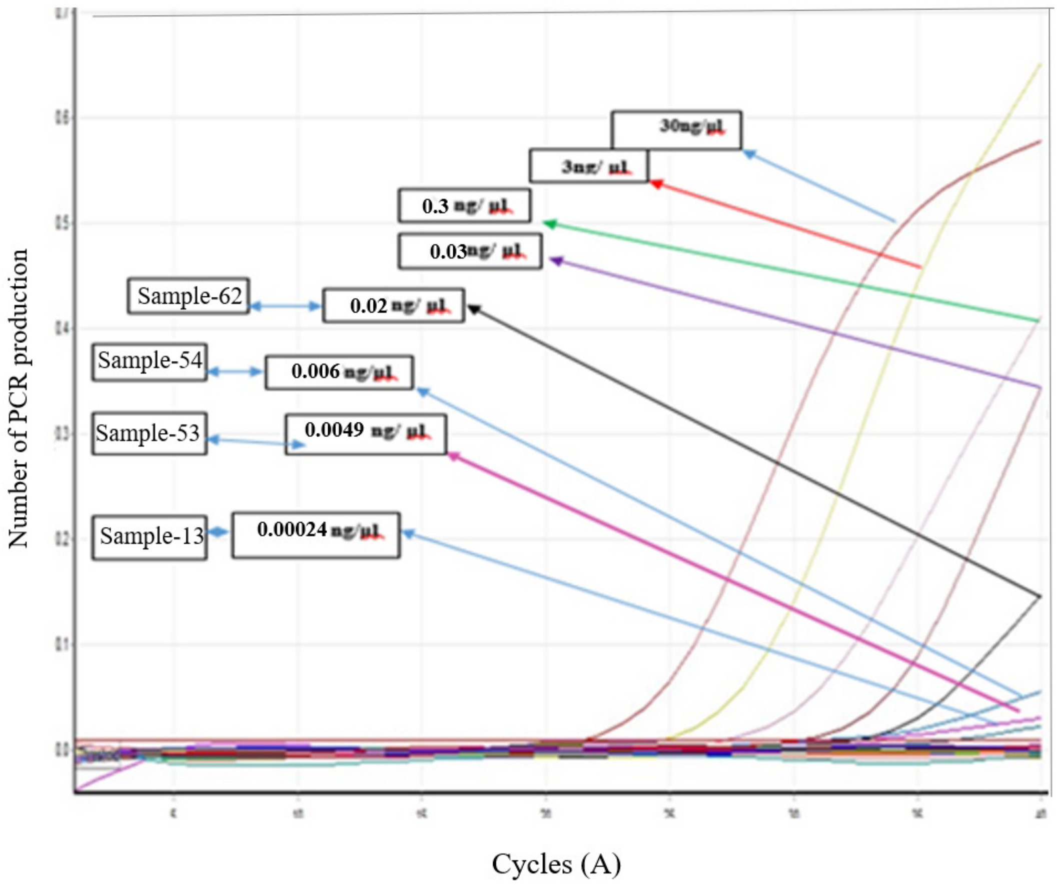

Figure 6. Amplification curve (A) and melting curve (B) for dilutions prepared from the positive control (*B. abortus*) and positive samples for *bcsp31* gene

Note: Sample 13-0.00024 ng/µL (green curve), Sample 53–0.0049 ng/µL (pink curve), Sample 54–0.006 ng/µL (blue), Sample 62-0.02 ng/µL (gray curve), 0.03 ng/µL (brown curve), 0.3 ng/µL (light brown), 3 ng/µL (yellow) and 30 ng/µL (purple curve).

milk and up to 18% in cheese. Differences likely reflect variations in sample size, livestock vaccination coverage, and hygienic practices. Internationally, prevalence has been reported as high as 40% in some African regions, while many European countries have achieved elimination through strict control programs.

This disease can be transmitted to humans through contaminated dairy products or contact with infected animals [13-15].

Table 3. The correlation between the quantity of bacteria in 1 mL of culture (CFU/mL) and the concentration of DNA

No.	Quantity of DNA (ng)	Quantity of Bacteria (CFU/mL)	Dilution
1	23/42	1.5×10^7	0.1
2	20/485	1.5×10^6	0.01
3	18/73	1.5×10^5	0.001
4	15/475	1.5×10^4	0.0001
5	12/94	1.5×10^3	0.00001
6	10/23	1.5×10^2	0.000001
7	8/23	1.5×10^1	0.0000001
8	6/34	1.5	0.00000001

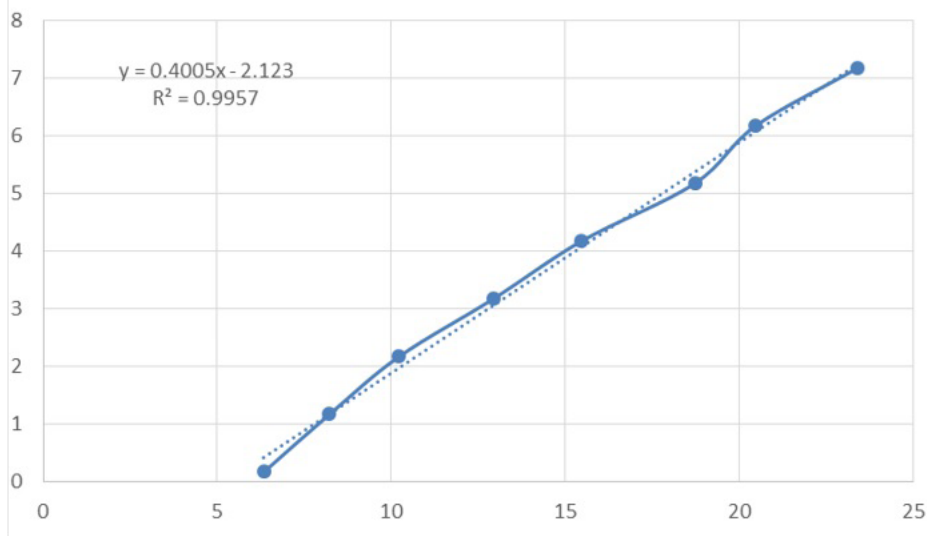

Figure 7. The standard curve of dilution of *Brucella* spp.

Multiple investigations in Iran have explored the presence of *B. abortus* and *B. melitensis* in raw cow's milk and dairy products to assess potential risks for consumers. In a study examined 238 unpasteurized dairy products from Shiraz province. 5.04% of the products were contaminated. Contamination was found in 18.75% of raw milk and 6.25% of yogurt samples. Cheese, dough, and traditional ice cream samples were contamination-free. Some of the contaminated samples contained *B. abortus*, *B. melitensis*, or both [16,17]. A study in Sarab, East Azerbaijan, Iran, found that 2.2% of 1000 cheese samples tested positive for *B. melitensis* and *B. abortus*. Similarly, research conducted in Toisarkan, Hamedan, Iran, identified 21 milk samples contaminated with *B. abortus* and 18 samples with *B. melitensis*. Additionally, in Isfahan and Chaharmahal and Bakhtiari, Iran, screenings revealed the existence of 1% *B. abortus* in raw cow's milk, local cheese (comprising 2.5% *B. abortus*, *B. melitensis*), and traditional cream (containing 1% *B. abortus*); in contrast traditional ice cream samples showed no contamination [18,19].

A semi-nested PCR method was employed to identify the presence of *Brucella* bacteria in samples of raw milk and cheese, revealing differing levels of contamination. The rates of *Brucella* contamination in various dairy products were as follows: 45.5% in raw goat milk, 39.1% in unpasteurized cheese, 27.3% in raw sheep milk, 26.3% in raw cow milk, 25% in pasteurized cheese, and 14.7% in pasteurized milk [16]. A study in Kurdistan, Iran, by Shafei et al. (2012) reported that 33.33% of 60 raw cow's milk samples were infected with *Brucella* spp., with 45% of those samples being *B. abortus* [19]. The prevalence of Brucellosis in Kurdistan was associated with its proximity to neighboring countries like Iraq and Turkey, leading to the introduction of non-native strains. Positive *Brucella* spp. samples were also identified in milk samples through PCR in Kerman, Iran. Another study in Lorestan, Iran, in 2017 found a 10% prevalence of *Brucella* spp. in 120 milk samples. *Brucella* spp. are found in raw milk in Sudan and Kenya with prevalence rates of 22.4% and 40%, and 18.9% and 65.5%, respectively. In Iraq, the prevalence of *Brucella* spp. infection varies between 8.4% and 56%, as determined through blood testing. European countries have successfully eliminated Brucellosis or have kept the disease prevalence low. Discrepancies in results from these studies can be attributed to methodological differences,

Table 4. The equation concentration of DNA in positive samples with the bacterial counts (CFU/g)

Sample-code	DNA Concentration (ng/µL)	Species	Bacteria Count (CFU/g or mL)
Cheese rind- 13	0.72	*B. abortus*	1>
Raw milk- 53	4.47	*B. melitensis*	8
Traditional ice cream- 54	7.59	*B. abortus*	8
Cheese rind - 62	62.94	*B. abortus*	>107

sample sizes, regional factors, livestock vaccination, and disease control measures [20-24].

Recent studies have shown that PCR is more sensitive and accurate in detecting *Brucella* spp. in various food items compared to traditional methods. One study found that 75% of samples tested positive for *B. abortus*, suggesting a high utilization of cow's milk in dairy production. Increased consumption of milk and dairy products can elevate the risk of Brucellosis, with research indicating that the infectious dose for *Brucella* spp. in food products ranges from 10 to 100 CFU/g/mL. The SYBR Green Real-Time PCR method used in a recent study could detect less than one CFU/g/mL of the product, demonstrating higher accuracy and sensitivity than previous methods [24]. In Semnan, Iran, *B. abortus* and *B. melitensis* have been detected in raw milk and traditional dairy products, indicating their presence in the local livestock populations. Regions that consume unpasteurized milk are at risk of *Brucella* spp. transmission, which poses a significant public health concern. Real-time PCR method with Sayber Green dye is advised for its precision and efficiency in detecting *Brucella* spp. During the spring and summer months, 75% of the samples analyzed showed the presence of *B. abortus* and *B. melitensis*, aligning with the periods when infected animals are breeding and lactating. Using techniques like real-time PCR for *Brucella* spp. detection in dairy products can improve pathogen identification accuracy. Public health strategies should emphasize the vaccination of livestock, strict control of raw milk sales, and consumer education about the risks of unpasteurized dairy consumption. Seasonal monitoring is also critical, as higher prevalence often coincides with breeding and lactation periods.

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Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

Data availability

The data that support the findings of this study are available upon request from the corresponding author.

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Authors' contributions

Conceptualization, study design, and supervision: Mahnoosh Parsaeimehr; Sample collection and execution of tests: Ali Nademirad and Hamid Staji; Data analysis and data interpretation: Hamid Staji and Ashkan Jebellijavan; Statistical analysis: Ashkan Jebellijavan; Writing: Mahnoosh Parsaeimehr and Marzieh Heidarieh; Writing: Mahnoosh Parsaeimehr and Marzieh Heidarieh; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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