1
Detection of *Brucella* spp. in raw milk and dairy products of traditional
2
domestic dairy sale centers by Real-Time PCR in Semnan

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ABSTRACT
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15
Introduction*:* *Brucellosis*, also known as Malta fever, is a significant zoonotic infectious disease
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in Iran, with considerable public health and economic complications. Human infection is primarily
17
acquired through the consumption of unpasteurized milk and dairy products. Conventional
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diagnostic approaches, such as culture and serology, are limited by low sensitivity, biosafety risks,
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and potential cross-reactivity. Molecular methods, particularly Real-Time PCR, offer greater
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sensitivity and specificity.
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Objectives: This cross-sectional study, investigated the presence of *Brucella* spp. In raw milk and
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traditional dairy products (cream, cheese, and ice cream) collected from local markets in Semnan,
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Iran.
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Material and Methods: A total of 95 samples were analyzed using Real-Time PCR with SYBR
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Green dye, targeting the bcsp31 gene for genus-level detection and species-specific primers for
26
Brucella abortus and *Brucella melitensis*. Also, the limit of detection (LOD) was evaluated using
27
the serial dilution of *Brucella* standard strain. Results: Results revealed that among the samples
28
tested, four samples were positive for *Brucella* spp. specifically, three samples were confirmed
29
Brucella abortus, and one sample was identified as *Brucella melitensis*. Following propagation,
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DNA fragments of 498 bp and 731 bp, corresponding to *Brucella abortus* and *Brucella melitensis*,

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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 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.

Keywords: *Brucella abortus*, *Brucella melitensis*, Real-Time PCR, Traditional dairy products

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]. *B. melitensis* and *B. 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 primary 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]. Food borne diseases are an important public health problem as it not only affects human health, but also has 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 food borne 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 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 informal distribution of raw dairy products, particularly in rural communities with limited awareness of hygienic practices. spp. [6]. Conventional diagnostic methods such as Culture are time-consuming, hazardous, and often yield false negatives due to the fastidious nature of the

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63 pathogen. Serologic methods, while rapid, may lack specificity because of cross-reactivity and
64 insufficient antibody levels. Molecular techniques, particularly polymerase chain reaction (PCR)
65 and Real -Time PCR (qPCR), provide superior sensitivity, specificity, and biosafety. Real-Time
66 PCR, [7,8].
67 The use of qPCR has provided several advantages over conventional PCR, such as quantification,
68 real-time, and in-situ analyses, in addition to automation. In this technique, the PCR products are
69 detected as they accumulate, and the amount of generated PCR product is proportional to the
70 increase in a signal fluorescent, which is monitored during the exponential phase. This technique
71 permits rapid identification and quantification of bacteria [9].
72 The present study aimed to investigate the presence of *Brucella spp*. In raw milk and traditional
73 dairy products sold in Semnan, Iran. Given the social, economic, and geographical conditions of
74 Semnan, where most people use traditional milk products, there is a possibility of people
75 contracting this disease. Using Real-Time PCR with SYBR Green dye, we targeted both genus-
76 specific genes to detect B. abortus and B. melitensis. The findings provide insights into the
77 prevalence of brucellosis in local dairy products and inform strategies for food safety and disease
78 control.
79

80 **2. Material and Methods**

81 **2.1. Samples preparation**

82 A cross-sectional study, involving three consecutive sampling rounds, was conducted over
83 approximately three months (early spring to late spring 2020) in traditional dairy products sold in
84 Semnan province, Iran. Based on a previously reported average prevalence of 6.6% for similar
85 products, a 95% confidence level (Z = 1.96), and a 5% margin of error, the sample size was
86 calculated using Cochran's formula, yielding 95 samples [6]. The total number of samples was
87 proportionally allocated to different dairy products according to their market sales volume,
88 resulting in 50 raw milk samples, 23 traditional ice cream samples, 11 traditional cream samples,
89 and 11 unpasteurized cheese samples, all collected randomly from traditional dairy sales centers.
90

91 **2.2. DNA extraction**

92 For each sample, 300µl was processed using the Dyna Bio DNA Mini Kit (Takapouzist Co., Iran)
93 according to the manufacturer's instructions. The purity and quantity of extracted DNA was

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94 measured by using a NanoDrop spectrophotometer (Thermo Scientific Nanodrop, Wilmington, 95 USA) at 260 and 280 nm (A260/280 ratio). Extracted DNA was eluted in 50-µl elution buffer. 96 Primers targeted the bcsp31 gene (223 bp fragment) for genus-level detection. Species-specific 97 primers targeted a 498 bp fragment (B. abortus, alkB gene) and a 731 bp fragment (B. melitensis, 98 BMEI 1162 gene). The specifications of the primers used are listed in Table 1. The primers 99 specificity was assessed by using BLAST [10].

100

101 Table 1- Oligonucleotide primers used for the detection of *Brucella* spp. *B. abortus* and *B. melitensis* 102 (Probert et al. 2004).

PCR Identification	Reverse primer	Forward primer	Fragment length
Brucella spp	GGGTAAAGCGTCGCCAGAAG	GCTCGGTTGCCAATATCAATGC	*223*
B.melitensis	CATGCGCTATGATCTGGTTACG	GCGGCTTTTCTATCACGGTATTC	*731*
B. abortus	CATGCGCTATGATCTGGTTACG	AACAAGCGGCACCCCTAAAA	*498*

103

104 **2.3. Real-Time PCR procedure**

105 Amplication was performed using a Rotor-Gene (Q MDx, Germany). The amplification reactions 106 contained 2 µL DNA template, 2.5 U AmpliTaq Gold DNA polymerase (Ampliqon, Copenhagen, 107 Denmark), 1X (5 µL of 10X) GeneAmp buffer II, 6 mM $MgCl_2$, 800 µM GeneAmp dNTP blend 108 (BioFact, Copenhagen, Denmark), 300 nM of each primer, and sterile water. The cycling 109 conditions utilized in this experiment were as follows: an initial denaturation step was conducted 110 at 95 °C for a duration of 10 minutes. This was succeeded by 40 cycles, which included 111 denaturation at 95 °C for 10 seconds, primer annealing at 55 °C for 30 seconds, and extension at 112 72 °C for 30 seconds. Subsequently, a melting curve analysis was executed over a temperature 113 range of 65 °C to 95 °C, with a transition rate of 0.1 °C/s, based on continuous fluorescence 114 measurements. Positive control contained the *Brucella strain* ATCC 23456 while negative control 115 contained nuclease free water. PCR efficiency was evaluated by constructing a standard curve 116 from serial dilutions of quantified *Brucella* control DNA. Cycle threshold (Ct) values were plotted 117 versus $\log_{10}$ initial copy number. The slope of the regression line was used to calculate efficiency 118 based on the equation:

119 $E=10^{-1/slope}-1$ [11, 12].

120 The limit of detection (LOD) was evaluated using the *Brucella* strain ATCC 23456, the standard 121 strain cultured in BHI broth overnight at 37°C and then adjusted to a turbidity equal to 0.5 122 McFarland (0.08–0.1 absorbance at 600nm wavelength) standard tube. Then, eight standards (S1–

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S8) were prepared by 10-fold serial dilutions from 1 to 0.00000001 dilutes of adjusted BHI broth (equal to 0.5 McFarland). Genomic DNA was extracted from all standards and quantitative real-time PCR was performed. Then, 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 value of 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 [11, 12].

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 non-parametric Kruskal–Walli's test, followed by Mann–Whitney U for pairwise comparisons. A p-value <0.05 was considered statistically significant.

3. Results

The Real-Time PCR technique was assessed through the analysis of the cycle threshold value (Ct), 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. three contained *Brucella abortus*, and one sample in raw milk contained *Brucella 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).

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	-	-

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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 (Fig. 1).

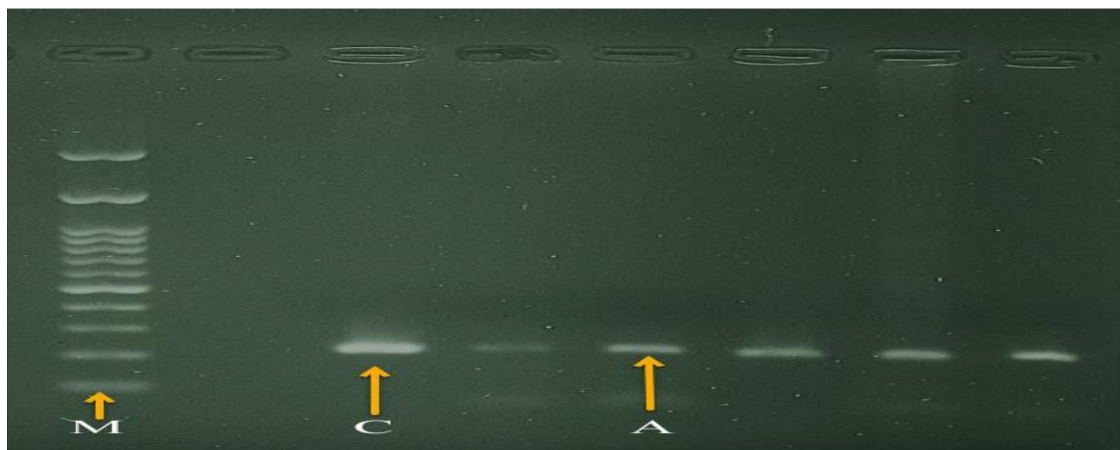

Figure 1. Agarose gel electrophoresis of PCR products generated from *Brucella* spp. at 55.3 °C. Lane M: marker (100 bp ladder). Lane C: positive control, Lane A: amplified bcsp31 fragment (233 bp). Arrows indicate the expected amplicon bands.

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

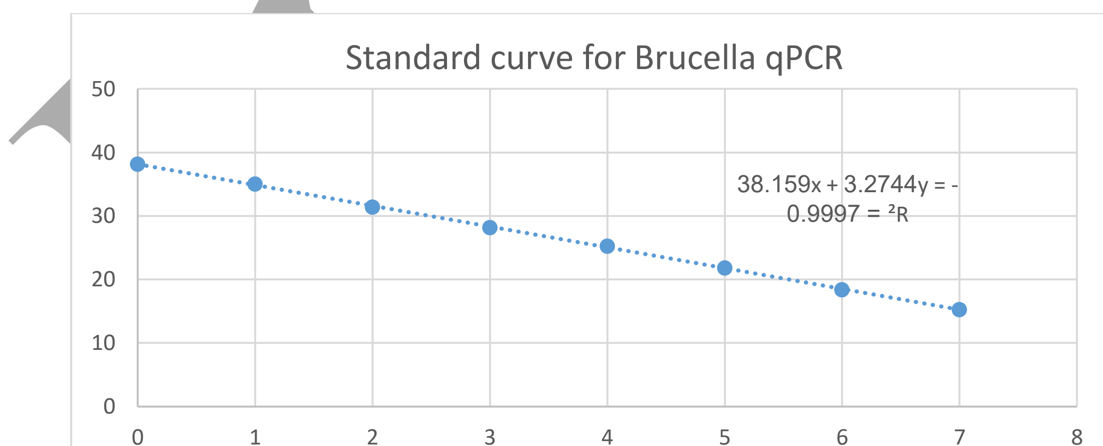

Figure 2: Standard curve for *Brucella* spp. qPCR assay ($\log_{10}$ copies vs. Ct) Slope = −3.27, $R^2 = 0.9997$, Efficiency = 102.02 %

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

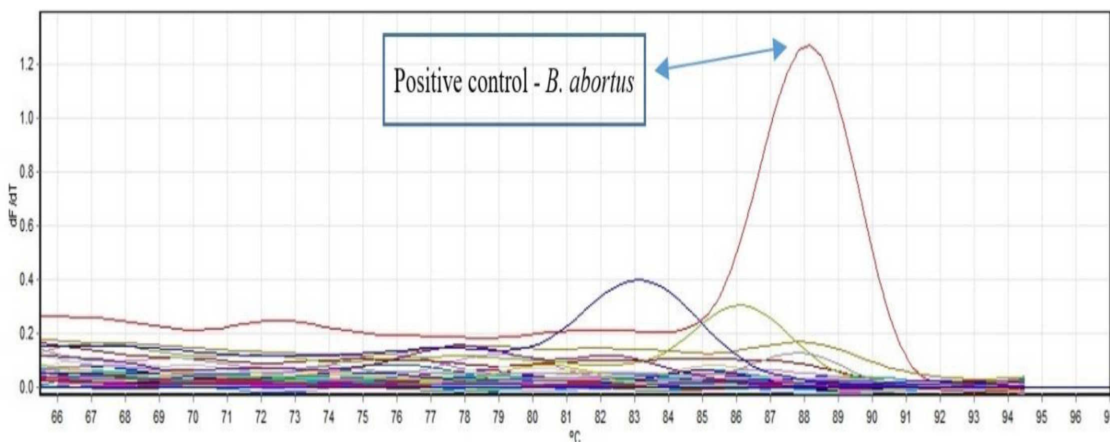

Figure 3. Melting peaks (left) and curves (right) of *Brucella* spp. positive samples in milk and dairy product samples, Target Tm of about 86.5±2.5 ° C (right). Melting peaks (left) and curves (right) of *Brucella* spp. positive samples in milk and dairy product samples, Target Tm of about 86.5±2.5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

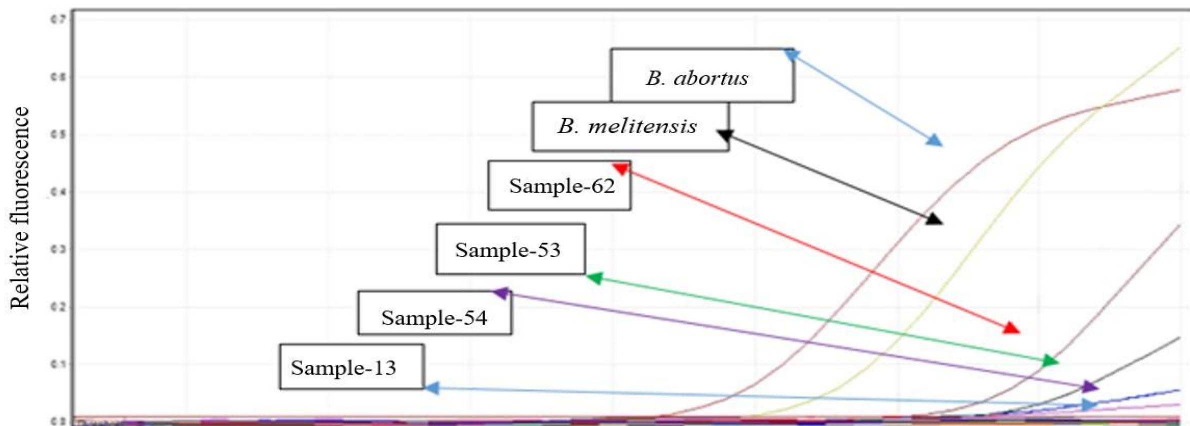

Figure 4. Proliferation curves: *Brucella abortus* (purple curve) and *Brucella melitensis* (yellow curve). *B. abortus:* Sample 62 (cheese rind) is shown by the brown curve, and Sample 13 (cheese rind) is represented by the pink curve. B. *melitensis*: Sample 54 (traditional ice cream) is dark blue curve and Sample 53 (raw milk) is gray curve.

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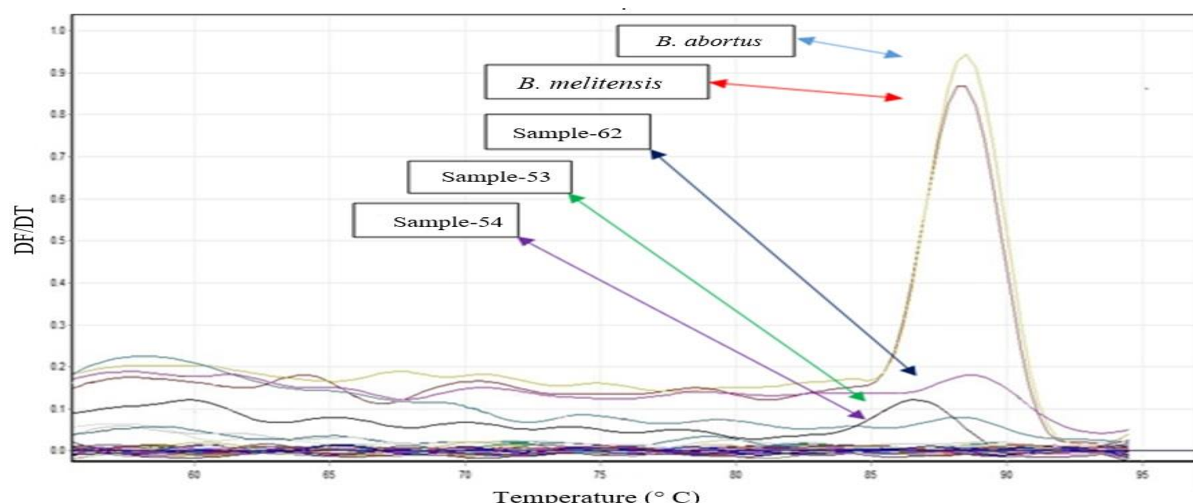

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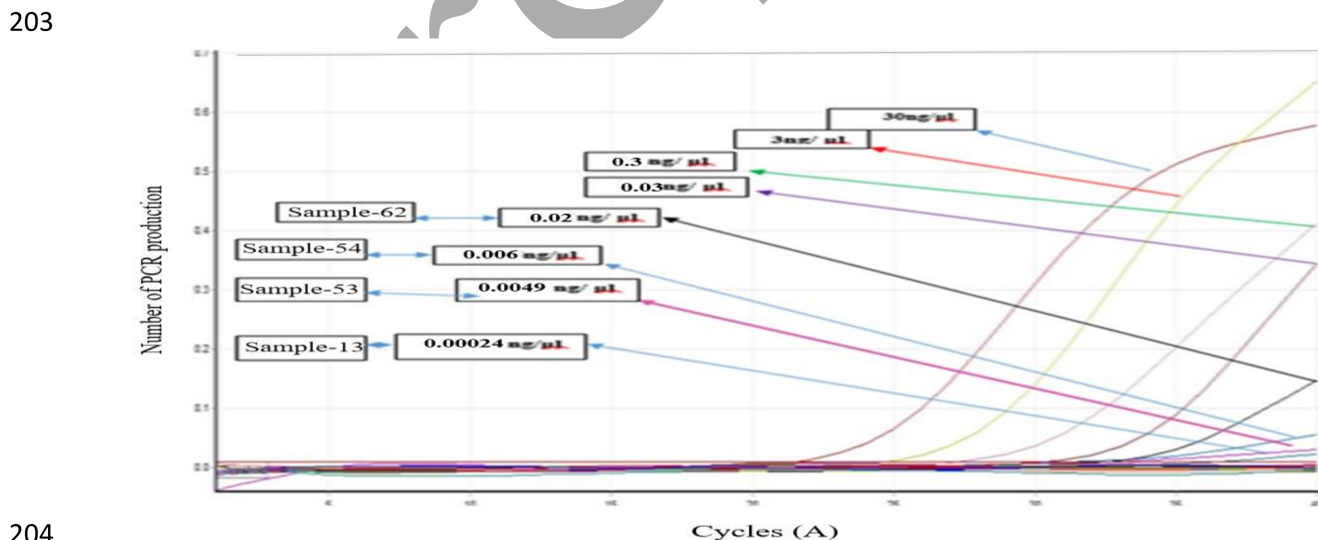
192 **Figure 5.** Melting curves of *Brucella* spp. identification for BMEI1162 and alkB genes. *B. abortus* (yellow curve)*:*
193 Sample 62 (cheese rind) is shown by the pink curve. B. *melitensis* (purple curve): Sample 54 (traditional ice cream)
194 is gray curve, Sample 53 (raw milk) is blue curve.

195

196 3.3. Quantification of *Brucella* spp. DNA concentrations in positive samples using a standard 197 curve

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

203



204

205 **Figure 6.** Amplification curve (A) and Melting curve (B) for dilutions prepared from the positive control
206 (*Brusella abortus*) and positive samples for bcsp31 gene. Sample 13 -0.00024 ng/µl (green curve), Sample

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207 53 – 0.0049 ng/µl (pink curve), Sample 54 – 0.006 ng/µl (blue), Sample 62-0.02 ng/µl (gray curve), 0.03
208 ng/µl (brown curve), 0.3ng/µl (light brown), 3 ng/µl (yellow) and 30 ng/µl (purple curve).
209
210
211
212
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214 **3.4. The equation concentration of DNA in positive samples with the bacterial counts**
215 **(cfu/gram or cc)**

216

217 A comparative analysis was performed utilizing a NanoDrop ND-1000 UV-Vis's
218 spectrophotometer (Thermo Scientific Nanodrop, Wilmington, USA), as outlined in Table 3, reveals
219 a significant correlation between colony-forming units per milliliter (cfu/ml) and the yield of DNA
220 obtained from standardized dilutions. This correlation is quantitatively represented by the equation
221 derived from the standard curve depicted in Figure 7. Consequently, the quantification of DNA
222 extracted from the positive samples was executed based on their positions along this standard
223 curve, and the results are summarized in Table 4.
224 Standard curves demonstrated strong linearity between DNA concentration and bacterial counts
225 (R^2 >0.99). The DNA concentration of positive samples ranged from 0.72 to 62.94 ng/ µl,
226 corresponding to bacterial loads of 1-10^7 cfu/ml. cheese rined samples showed the highest bacterial
227 counts (>10^7 cfu/g).

228
229

230 Table 3- The correlation between the quantity of bacteria in 1 mL of culture (cfu/mL) and the concentration
231 of DNA
232

Number	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

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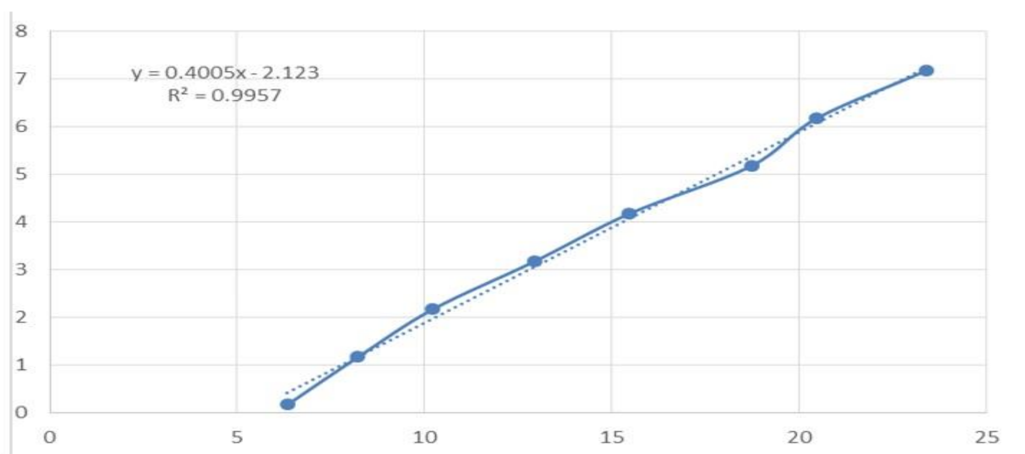

236 7

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

238

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

240

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*	>10^7

241

242 **4. Discussion**

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

247 Our findings align with previous studies in Iran, where contamination rates varied wildly
248 depending on product type, region, and diagnostic methods. For examples, studies have reported
249 contamination rates of 2-10% in milk and up to 18% in cheese. Differences likely reflect variations
250 in sample size, livestock vaccination coverage, and hygienic practices. Internationally, prevalence
251 has been reported as high as 40% in some African regions, while many European countries have
252 achieved elimination through strict control programs.

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

255 Multiple investigations in Iran have explored the presence of *B. abortus* and *B. melitensis* in raw
256 cow's milk and dairy products to assess potential risks for consumers. In a study examined 238
257 unpasteurized dairy products from Shiraz province. 5.04 % of the products were contaminated.
258 Contamination was found in 18.75% of raw milk and 6.25% of yogurt samples. Cheese, dough,
259 and traditional ice cream samples were contamination-free. Some of the contaminated samples had
260 *B. abortus*, *B. melitensis*, or both [16,17]. A study in Sarab, East Azerbaijan, Iran, found that 2.2%

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261 of 1000 cheese samples tested positive for *B. melitensis* and *B. abortus*. Similarly, research
262 conducted in Toisarkan, Hamedan, Iran, identified 21 milk samples contaminated with *B. abortus*
263 and 18 samples with *B. melitensis*. Additionally, In Isfahan and Chaharmahal and Bakhtiari, Iran,
264 screenings revealed the existence of 1% *B. abortus* in raw cow's milk, local cheese (comprising
265 2.5% *B. abortus*, *B. mellitensis*), and traditional cream (containing 1 % *B. abortus*); In contrast
266 traditional ice cream samples showed no contamination [18,19]. A semi-nested PCR method was
267 employed to identify the presence of *Brucella* bacteria in samples of raw milk and cheese,
268 revealing differing levels of contamination. The rates of *Brucella* contamination in various dairy
269 products were as follows: 45.5% in raw goat milk, 39.1 % in unpasteurized cheese, 27.3% in raw
270 sheep milk, 26.3% in raw cow milk, 25 % in pasteurized cheese, and 14.7 % in pasteurized milk
271 [16]. A study in Kurdistan, Iran, by Shafei et al. (2012) reported that 33.33% of 60 raw cow's milk
272 samples were infected with *Brucella* spp., with 45 % of those samples being *B. abortus* [19]. The
273 prevalence of *Brucellosis* in Kurdistan was associated with its proximity to neighboring countries
274 like Iraq and Turkey, leading to the introduction of non-native strains. Positive *Brucella* spp.
275 samples were also identified in milk samples through PCR in Kerman, Iran. Another study in
276 Lorestan, Iran, in 2017 found a 10 % prevalence of *Brucella* spp. in 120 milk samples. *Brucella*
277 spp. are found in raw milk in Sudan and Kenya with prevalence rates of 22.4 % and 40 %, and
278 18.9 % and 65.5 %, respectively. In Iraq, the prevalence of *Brucella* spp. infection varies between
279 8.4 % and 56 % as determined through blood testing. European countries have successfully
280 eliminated Brucellosis or have kept the disease prevalence low. Discrepancies in results from these
281 studies can be attributed to differences in methodological differences, sample sizes, regional
282 factors, livestock vaccination, and disease control measures [20-24].
283 Recent studies have shown that PCR is more sensitive and accurate in detecting *Brucella* spp. in
284 various food items compared to traditional methods. One study found that 75 % of samples tested
285 positive for *B. abortus*, suggesting a high utilization of cow's milk in dairy production. Increased
286 consumption of milk and dairy products can elevate the risk of Brucellosis, with research
287 indicating the infectious dose for *Brucella* spp. in food products ranges from 10 to 100 cfu/g/ml.
288 The SYBR Green Real-Time PCR method used in a recent study could detect less than one
289 cfu/g/ml of the product, demonstrating higher accuracy and sensitivity than previous methods.
290 [24]. In Semnan, Iran, *B. abortus* and *B. melitensis* have been detected in raw milk and traditional
291 dairy products, indicating their presence in the local livestock populations. Regions that consume
292 unpasteurized milk are at risk of *Brucella* spp. transmission, which poses a significant public health
293 concern. Real-Time PCR method with Sayber Green dye is advised for its precision and efficiency
294 in detecting *Brucella* spp. During the spring and summer months, 75% of the samples analyzed
295 showed the presence of *B. abortus* and *B. melitensis*, aligning with the periods when infected
296 animals are breeding and lactating. Using techniques like Real-Time PCR for *Brucella* spp.
297 detection in dairy products can improve pathogen identification accuracy. Public health strategies
298 should emphasize vaccination of livestock, strict control of raw milk sales, and consumer
299 education about the risks of unpasteurized dairy consumption. Seasonal monitoring is also critical,
300 as higher prevalence often coincides with breeding and lactation periods.

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Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

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Data Availability

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

Authors' Contributions

MP, who also played a pivotal role in its design, initiated the conceptualization of the study. ANR and HS were responsible for the collection of samples and the execution of tests. HS and AJJ contributed to the analysis of data and the interpretation of results. AJJ was responsible for the statistical analysis. MP provided supervision for the project. MP and MH were responsible for the drafting of the manuscript and the review of the literature. All authors have read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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