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Prevalence of *Brucella* Contamination in Unpasteurized Dairy Products in Sistan Region, Iran, Using PCR Assay

K. Mehdipour[a], D. Saadati[b,*], M. Najimi[c], A. Hosseini[d]

[a] DVM graduated, Faculty of veterinary medicine, university of Zabol, Zabol, Iran.
[b] Department of Food Hygiene, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran.
[c] Department of Pathobiology, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran.
[d] Zabol veterinary office, Sistan and Baluchestan provincial veterinary service, Iranian veterinary organization, Zabol, Iran.

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Abstract

Brucellosis is one of the most widespread zoonotic diseases globally, posing a significant public health and economic burden. It is primarily transmitted to humans through the consumption of contaminated milk and raw dairy products, or by direct contact with infected animals. This study aimed to determine the prevalence of *Brucella* contamination in unpasteurized dairy products sold in the Sistan region, Iran, utilizing the Polymerase Chain Reaction (PCR) method. A total of 175 unpasteurized traditional dairy samples were collected from various distribution centers in Zabol city. These samples included 35 samples each of milk, yogurt, cheese, buttermilk, and ice cream. DNA extraction was performed using the boiling method. Following DNA extraction, PCR was conducted to detect the presence of *Brucella* bacteria and identify the species, specifically targeting *Brucella abortus* and *Brucella melitensis*. The results revealed that only one sample (0.58% of 175 tested samples) was positive for *Brucella*. This positive sample was identified as *Brucella abortus*, and it originated from one of the 35 milk samples analyzed, indicating a prevalence of 2.86% in unpasteurized milk samples. This low prevalence of *Brucella* infection in the Sistan region, compared to findings from other past studies in the area, is likely attributable to the recent, systematic, and annual free vaccination campaigns against brucellosis in livestock throughout the Sistan region. These findings highlight the effectiveness of ongoing vaccination efforts in reducing the spread of brucellosis in the region.

1. Introduction

Brucellosis, also known as Malta fever or undulant fever, is classified by the World Health Organization (WHO) as one of the seven neglected zoonotic diseases (Hull and Schumaker 2018). This bacterial infection significantly impacts public health and animal production globally, particularly in developing countries where it perpetuates poverty. While many developed nations have successfully eradicated this important zoonosis (Ning *et al.*, 2018), brucellosis remains widespread in regions such as the Mediterranean, Middle East, Central Asia, and parts of Latin America, making it a global health concern (Maryam *et al.*, 2025; Zhi-guo *et al.*, 2023; Qureshi *et al.*, 2023). It is estimated that 1.6–2.1 million new human cases occur

*Corresponding author: D. Saadati (Assistant Professor)
E-mail address: saadatidariush@gmail.com
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annually worldwide, with infected animals and their products being the most probable sources of infection (Qureshi *et al.*, 2023).

Brucella spp. are primarily transmitted to humans through the consumption of unpasteurized dairy products or by direct contact with infected animals or their secretions (Khurana *et al.*, 2020). Among the various species, Brucella melitensis and B. abortus are the most frequently reported in human and animal infections in Iran, where they pose major public health and economic challenges (Maryam and Saeid 2017). B. melitensis, typically associated with sheep and goats, is considered the most virulent species in humans, while B. abortus is mainly linked to cattle. Infections in humans often present with nonspecific symptoms such as fever, fatigue, and joint pain, making clinical diagnosis difficult without laboratory confirmation (Bin *et al.*, 2025; Lai, Qiulan, and Zhongjie 2021).

The Sistan region, located in southeastern Iran, is particularly vulnerable to brucellosis due to its high reliance on traditional, unpasteurized dairy production and close contact between livestock and humans. Although national vaccination campaigns have been implemented in recent years to control the disease, sporadic human and animal cases are still reported (Narouei *et al.*, 2022; Ahmadi *et al.*, 2021). These observations highlight the importance of continuous epidemiological surveillance and the need for reliable diagnostic tools to monitor infection sources in high-risk areas.

In this context, the present study aims to evaluate the prevalence of Brucella contamination in unpasteurized dairy products collected from the Sistan region using the Polymerase Chain Reaction (PCR) technique. PCR is recognized as a highly sensitive and specific molecular method for the detection of Brucella DNA, allowing not only for rapid identification but also for species-level differentiation (Moussa *et al.*, 2011). The findings of this study may provide insight into the effectiveness of ongoing vaccination programs and inform public health strategies to reduce the burden of brucellosis in endemic areas.

2. Materials and Methods

2.1. Study Area and Sample Population

This study was conducted in the Sistan region, focusing on dairy products distributed in Zabol city. The target population included unpasteurized dairy products sold by local vendors, and sampling was conducted in 2023.

2.2. Sample Collection and Storage

A total of 175 samples of traditional unpasteurized dairy products were collected from various distribution centers across Zabol city. The samples included milk, yogurt, cheese, buttermilk, and ice cream, with 35 samples collected for each product type. Samples were collected using 15 ml Falcon tubes and immediately transported to the laboratory of the Faculty of Veterinary Medicine, University of Zabol, in a cold box containing ice. All equipment, including ampoules, tips (yellow, blue, white), and Eppendorf tubes, were sterilized by autoclaving at 121°C and 1.5 atmospheres for 15 minutes before use. Upon arrival at the laboratory, samples were stored at -20°C until DNA extraction.

2.3. DNA Extraction

DNA extraction from all samples was performed using the boiling method. Initially, 1 ml of each sample was transferred into 1.5 ml Eppendorf tubes and centrifuged at 3500 rpm for 5 minutes. After centrifugation, the supernatant was discarded and replaced with 1 ml of sterile distilled water. The samples were centrifuged again under the same conditions, and the resulting supernatant was removed. Subsequently, 100 μl of sterile distilled water was added to each tube, and the contents were mixed thoroughly using a vortex mixer. The tubes were then incubated in a thermocycler at 98°C for 10 minutes to facilitate DNA release. After incubation, the samples were centrifuged at 14,000 rpm for 10 minutes, and the supernatant containing the extracted DNA was carefully collected using a micropipette. The extracted DNA was stored at -18°C until further analysis by PCR.

2.4. PCR Assay

2.4.1. Primer Synthesis and Preparation

Previously validated primer sets were employed for the detection of Brucella species. The synthesis of primers was carried out by Pishgam Biotech Company. For the initial genus-level screening, the bcsp31 gene was targeted using the primers B4 (5′-TGG CTC GGT TGC CAA TAA CAA-3′) and B5 (5′-CGC GCT TGC CTT TCA GGT CTG-3′), which produced an amplicon of 223 base pairs (Baily *et al.*, 1992). To identify Brucella abortus (biovars 1, 2, and 4) and Brucella melitensis at the species level, multiplex PCR was performed. For the amplification of B. abortus, the primers Ba-SP (5′-GAC GAA CGG AAT TTT TCC AAT CCC-3′) and IS711SP (5′-TGC CGA TCA CTT AAG GGC CTT CAT-3′) were used, generating a 498 bp fragment. Detection of B. melitensis was achieved using the Bm-SP primer (5′-AAA TCG CGT CCT TGC TGG TCT GA-3′) in combination with IS711SP, resulting in a 731 bp amplicon (Bricker and Halling 1994).

The primers were reconstituted from lyophilized form following the manufacturer's protocol by dissolving them in sterile deionized water to obtain a stock concentration of 100 pmol/μl. Working solutions with a final concentration of 10 pmol/l were prepared for

routine use and stored at −20°C for long-term preservation.

The final PCR reaction mixture had a total volume of 25 μl and consisted of 12.5 μl of PCR master mix, 9.5 μl of deionized water, 1 μl each of forward and reverse primers, and 1 μl of DNA template.

2.4.2. PCR Cycling Conditions

The thermal cycling protocol used for amplification of the bcsp31 gene as well as for species-specific detection of Brucella abortus and Brucella melitensis followed standardized conditions. The PCR program began with an initial denaturation step at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 1.5 minutes, annealing at 56°C for 2 minutes, and extension at 72°C for 2 minutes. A final extension step was carried out at 72°C for 5 minutes to complete the reaction.

2.5. Gel Electrophoresis and Visualization

After PCR amplification, the products were confirmed using horizontal electrophoresis on a 2% agarose gel. A 100bp-3kbp DNA ladder was used as a size marker. 5 μl of each PCR product was loaded onto the gel. The gel was stained with ethidium bromide and visualized under UV light using a Gel Documentation system. Ethidium bromide is a carcinogen, so appropriate safety precautions (gloves, mask, and lab coat) were observed.

2.6. Data Analysis

The prevalence of *Brucella* contamination in the sample population was calculated using a binomial distribution with 95% confidence intervals.

3. Results

Out of the 175 unpasteurized dairy samples examined in this study, only one sample (0.58%) (With 95% CI from 0.01% to 3.1%) tested positive for *Brucella*. This positive sample was specifically milk, and the identified species was *Brucella abortus*. This translates to a prevalence of 2.86% within the 35 milk samples analyzed.

The positive results were confirmed by the presence of specific DNA bands for the *Brucella* genus and *Brucella abortus* species using the respective primer sets.

This observed prevalence of *Brucella* infection in unpasteurized dairy products in the Sistan region is significantly lower compared to historical data and other studies conducted in the past years in the same region. For instance, a 2018 study detected *Brucella melitensis* in 35% of aborted sheep fetuses in the Sistan region. Another study in Sistan reported a seroprevalence of 2.3% for *Brucella* in sheep and goats, with PCR showing a 17% positivity rate in 2021.

The current low prevalence is strongly attributed to the sustained and systematic free brucellosis vaccination programs for livestock that have been implemented annually in the Sistan region in recent years.

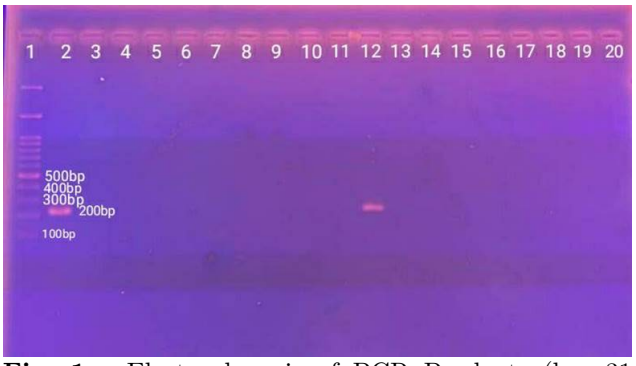

Fig. 1. Electrophoresis of PCR Products (bcsp31 gene) showing *Brucella* genus amplification (This image would show a band at 223 bp for the positive sample, indicating the presence of *Brucella* DNA)

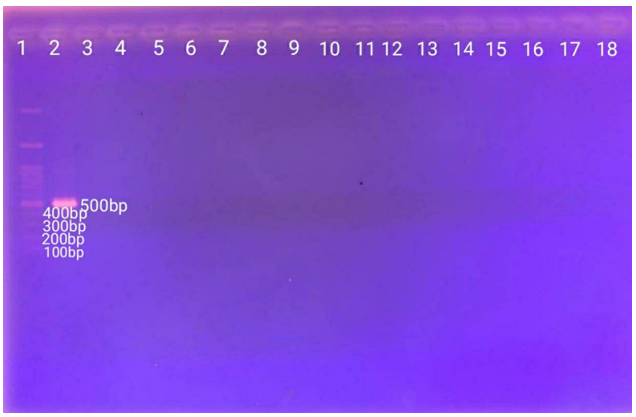

Fig. 2. Electrophoresis of PCR Products (Ba-SP and IS711 primers) showing *Brucella abortus* species amplification (This image would show a band at 498 bp for the positive sample, confirming *Brucella abortus*)

4. Discussion

The findings of this study, revealing a very low prevalence of *Brucella* contamination (0.58%) in unpasteurized dairy products in the Sistan region, and specifically *Brucella abortus* in only one milk sample, are noteworthy and encouraging. This outcome stands in stark contrast to the higher prevalence rates reported in historical studies within Iran and internationally.

Previous research in Iran has consistently shown higher rates of brucellosis. For example, Jawadi *et al.*, reported a seroprevalence of 4.53% in sheep and 5.33% in goats in East Azerbaijan province (Javadi *et al.*, 2007). Kaboutari *et al.* found a 6.4% seroprevalence in sheep and goats and a 25.3% flock level prevalence in southern Kerman province (Kaboutari *et al.*,

2015). Mohammadi et al. detected *Brucella melitensis* in 17.5% of aborted sheep fetuses in Gonbad-e Qabus (Mohammadi *et al.*, 2019). In Zabol itself, a 2016 study found that 7% of cow milk samples tested positive for *Brucella abortus* and *Brucella melitensis*, with 5% being *B. abortus* positive and 2% *B. melitensis* positive (Ahmadi *et al.*, 2021). Even in a 2017 study specifically on sheep and goats in the Sistan region, a PCR-based prevalence of 11.3% for *Brucella* was reported, with all positive samples being *B. melitensis*. These comparisons underscore the significant reduction observed in the current study (Narouei *et al.*, 2022; Ahmadi *et al.*, 2021).

One of the most important factors contributing to the reduced prevalence of brucellosis observed in the present study appears to be the implementation of regular phases of free vaccination campaigns against brucellosis in livestock across the Sistan region in recent years. These government-supported programs, which impose no financial burden on animal owners, have likely played a key role in controlling the disease. Vaccination of animals is globally recognized as one of the most effective tools for preventing human brucellosis. Countries like Kuwait have demonstrated the efficacy of extensive animal vaccination programs in controlling brucellosis in cattle, sheep, and goats (Al-Khalaf, Mohamad, and Nicoletti 1992). Control strategies built upon vaccination, testing, and culling have proven successful in many developed countries (Ning *et al.*, 2018).

B. melitensis is typically the most prevalent species in sheep and goats and the primary cause of human brucellosis. Compared to other *Brucella* species, *B. melitensis* causes the most severe and acute form of the disease in humans and is considered the most pathogenic for humans (Patrycja *et al.*, 2018).

Brucella abortus is primarily associated with brucellosis in cattle. It is known to cause reproductive disorders such as abortion, infertility, and decreased milk production in infected animals. Although it is less pathogenic to humans compared to *Brucella melitensis*, it remains a zoonotic agent of concern, particularly in regions where cattle are raised alongside other ruminants (Dereje 2022).

In the present study, the detection of *B. abortus* in a milk sample-despite the predominance of sheep and goat dairy production in the region-suggests a possible bovine origin of contamination. This unexpected finding highlights the need for sustained surveillance not only in small ruminants but also in cattle, even in areas where bovine dairy farming is limited. It also emphasizes the risk of cross-species transmission and the importance of implementing comprehensive brucellosis control strategies that encompass all livestock

The study also reinforces the utility of PCR as a highly sensitive and specific diagnostic tool for *Brucella* detection, especially when the bacterial load in samples might be low. Unlike traditional serological methods that detect antibodies and can yield false positives due to cross-reactions or persistent antibodies post-treatment, PCR directly identifies bacterial DNA, making it reliable for diagnosing active infections and even low bacterial counts. This is particularly advantageous for milk samples where the presence of live *Brucella* poses a direct risk of transmission (Batrinou *et al.*, 2022; Ahmadi *et al.*, 2021).

5. Conclusion

Despite the positive trend, the presence of even a single *Brucella* positive sample in unpasteurized dairy products underscores the ongoing risk to public health, especially given the common practice of consuming raw dairy in the region. The “One Health” approach, integrating animal health and public health efforts, is crucial for sustained success in brucellosis control.

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