Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol.25 No.2 Ser. No.87 2024

IRANIAN JOURNAL OF VETERINARY RESEARCH

Short Paper

Detection of *Brucella* infection in raw milk of livestock in Famenin, West part of Iran

Adabi, M.[1, 2]; Gharekhani, J.[3]; Alamian, S.[4]; Varasteh Shams, M.[1]; Fathi Sheikhi, M.[5]; Shivapour, Z.[6]; Valiei, Z.[7]; Khazaiee, S.[8] and Ghaderi, H.[9*]

[1]Infectious Diseases Research Center, Hamadan University of Medical Sciences, Hamadan, Iran; [2]Infectious Ophthalmologic Research Center, Imam Khomeini Hospital Clinical Research Development Unit, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; [3]Department of Laboratory Sciences, Central Veterinary Laboratory, Iranian Veterinary Organization (IVO), Hamedan Veterinary Office, Hamedan, Iran; [4]Department of Brucellosis, Razi Vaccine and Serum Research Institute, Agriculture Research, Education and Extension Organization (AREEO), Karaj, Iran; [5]MSc in Health Education, Deputy of Health, Hamadan University of Medical Sciences, Hamadan, Iran; [6]MSc in Statistics, Faculty of Health, Hamadan University of Medical Sciences, Hamadan, Iran; [7]MSc in Microbiology, Department of Microbiology, Faculty of Basic Sciences, Hamadan Branch, Islamic Azad University, Hamadan, Iran; [8]Research Center for Health Sciences, Hamadan University of Medical Sciences, Hamadan, Iran; [9]Department of Microbiology, Faculty of Veterinary Sciences, Ilam University, Ilam, Iran

**Correspondence:* *H. Ghaderi, Department of Microbiology, Faculty of Veterinary Sciences, Ilam University, Ilam, Iran. E-mail: h.ghaderi@ilam.ac.ir*

10.22099/IJVR.2024.49557.7292

(Received 22 Feb 2024; revised version 12 Jun 2024; accepted 21 Jul 2024)

Abstract

Background: Timely diagnosis of brucellosis is the starting point for effective programs to control brucellosis in humans and animals. **Aims:** This study aimed to detect *Brucella* infection in milk samples from livestock in Famenin, an endemic region of western Iran, using a milk ring test and molecular techniques. **Methods:** In this cross-sectional study, 738 raw milk samples were randomly collected from cattle, sheep, and goats. Milk samples were screened using the milk ring test (MRT). In addition, polymerase chain reaction (PCR) method was applied to detect *Brucella* spp. in all MRT-positive samples. DNA from the milk samples was extracted and used for PCR using the *BCSP31* and *IS711* loci. **Results:** Of the samples tested using MRT, 46 (6.23%, 95% CI: 2.83-9.63%) yielded positive results. Of the 46 seropositive samples, 42 (91.30%) were from sheep and 4 (8.70%) were from goats, while no bovine samples had positive MRT results. PCR analysis confirmed that 78.26% (36/46) of MRT-positive samples belonged to the genus *Brucella*. Furthermore, 83.33% (30/36) of the confirmed samples were identified as *B. melitensis*, while 16.66% (6/36) were identified as *B. abortus*. **Conclusion:** The results obtained from MRT evaluation of milk samples did not align entirely with the findings of the molecular examinations. The PCR method has minimal biological contamination and high sensitivity and accuracy, especially for determining *Brucella* species. Raw milk should be routinely assessed for *Brucella* contamination. This work is necessary to identify hidden infections and break the chain of transmission of brucellosis.

Key words: Brucellosis, Food-borne diseases, Livestock, Milk ring test, PCR

Introduction

Brucellosis is a worldwide zoonotic infection caused by *Brucella* spp. (Khurana *et al*., 2021; World Health Organization, 2022). The prevalence of brucellosis in humans directly indicates the frequency of brucellosis in livestock (Akinyemi *et al*., 2022). Brucellosis, an occupational disease, is transmitted through direct or indirect contact with infected animal tissues and their secretions, consumption of contaminated animal products such as raw milk, dairy products, and meat, contact with infected placenta, or laboratory culture media (Dadar *et al*., 2019b; Bennett and Bronze, 2021). The udder tissue is an important site for *Brucella*, and infection of the udder is accompanied by steady or periodic excretion of the organism into the milk (Al-Afifi *et al*., 2022). The most common route of transmission is consumption of raw or unpasteurized milk. *Brucella* spp. can be isolated from dairy products of cattle, sheep, goats, and camels (Shakir, 2021). Although significant advances in brucellosis control techniques have been made in many countries, the infection persists in animal populations and can be transmitted to humans is likely (Bennett and Bronze, 2021).

In Iran, brucellosis is considered the main cause of financial and health problems in the livestock industry (Alamian *et al*., 2021; Dadar *et al*., 2021). The incidence of brucellosis is higher in some areas of Iran, such as Hamadan, Kermanshah, and Lorestan than in other areas. Famenin is an endemic area with a high prevalence of

brucellosis in Hamadan Province, western Iran (Keramat *et al*., 2020).

Serological tests are usually the most reliable method of diagnosis because the disease can be present with symptoms similar to those of other infectious diseases (Keramat *et al*., 2020; Bennett and Bronze, 2021). Although culture and isolation of bacteria is the gold standard for diagnosis of brucellosis, there are some limiting factors for *Brucella* spp. detection such as slow growth and the need for special media. Therefore, serological tests such as milk ring test (MRT), Rose Bengal plate test (RBPT), Wright serum agglutination test (AST), 2-mercaptoethanol (2ME), and enzyme-linked immunosorbent assay (ELISA) are recommended for the infection detection (Mantur *et al*., 2010; Al Dahouk and Nöckler, 2011; Adabi *et al*., 2021). MRT, a rapid agglutination test using the *Brucella abortus* antigen, is one of the most commonly used primary screening systems for detecting antibodies against *Brucella* in milk and dairy products (Khan *et al*., 2018).

Recently, rapid and reliable identification of *Brucella* species has been enabled by molecular methods with high sensitivity and specificity that minimize the risk of infection transmission. (Adabi *et al*., 2022). This study aimed to detect *Brucella* infections in raw milk samples from domestic animals (cattle, sheep, and goats) in Famenin, Hamedan Province, using MRT and molecular biology techniques.

Materials and Methods

Ethical approval

This study was approved by the Ethics Committee of Hamadan University of Medical Sciences, Iran (IR.UMSHA.REC.1399.7).

Study period and location

This study was conducted from September 2020 to March 2021 in Famenin, Hamadan Province. Hamedan is one of the western provinces in Iran (34.77° N and 48.58° E) (Fig. 1).

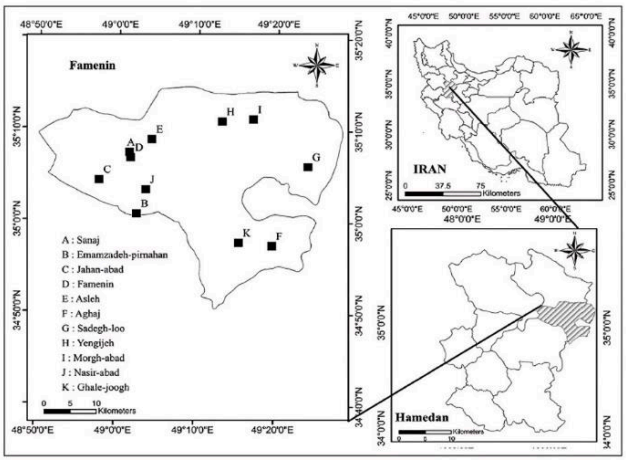

Fig. 1: The geographic map and distribution of sampling regions (n=11, A-K) in Famenin, Hamedan Province, west of Iran (16)

Study design and sampling

This study followed the animal testing phase (second phase) of the Famenin Brucellosis Cohort Study in 2019 (Adabi *et al*., 2022). Based on statistical analysis of the previous phase of the cohort and considering the estimated prevalence of 2.5% of animal brucellosis in the province, 1167 livestock, including 90 cows, 1660 sheep and goats should be examined; however, as many sheep could not be milked, they were excluded from the study. A total of 738 raw milk samples were collected from Famenin's livestock, including 98 cattle, 450 sheep, and 190 goats. All samples (in sterile 50 ml tubes) were collected and stored in ice packs and transferred to the brucellosis laboratory at Hamadan University of Medical Sciences.

MRT

Elementary screening for the presence of *Brucella* bacteria in all raw milk samples was performed by ring test with *Brucella abortus* antigen (Razi Vaccine and Serum Research Institute, Karaj, Iran). First, a 1.0 ml milk sample was dropped into an agglutination tube. The test was performed by mixing a drop (0.03 ml) of *Brucella* antigen (hematoxylin-stained antigen) into each tube. All samples, including negative and positive controls, were incubated at 37°C for 1 h. Agglutinated bacterial cells rise through fat globules and form a creamy layer on top of the tubes. The formation of a dark blue ring at the top of the tube was considered a positive reaction (Mohamand *et al*., 2014; Babaoglu *et al*., 2018). All MRT-positive samples were selected for molecular analysis.

Molecular identification

DNA extraction from raw milk

Based on the supplier's manual, DNA extraction from MRT-positive samples was performed using a DNA purification kit (Cinnaclone, Tehran, Iran, Cat. No. PR881613). The extracts were analyzed qualitatively and quantitatively using electrophoresis (in a 1% agarose gel) and NanoDrop (Eppendorf, Germany) by reading A260 and A280, respectively.

DNA amplification

The extracts were analyzed by polymerase chain reaction (PCR) using primers B4 and B5 (Table 1) to detect the genus *Brucella* (Garshasbi *et al*., 2014). The final volume of each reaction was 12.5 µL, including 6.25 µL of 2x PCR master mix (Amplicon, Denmark), 0.5 µL of each primer, 4 µL of DNA template, and distilled water up to 12.5 µL.

B. abortus and *B. melitensis* were detected using *IS711*-PCR (insertion sequence 711) (Dadar *et al*., 2019a). Although the forward primer of *IS711* is unique for detecting *Brucella* species, the reverse primers were derived from a specific locus on the chromosomes of *B. abortus* and *B. melitensis* (Table 1). A total volume of 12.5 µL was used for this reaction using the same *BCSP31* PCR mixture. The PCR profile was completed using a thermal cycler (T100 PCR Thermal Cycler,

Table 1: The characteristics of the primers used for the molecular detection of *Brucella*

Gene	Primer	Product	Reference
BCSP31-B4 *BCSP31*-B5	Forward: 5´ TGGCTCGGTTGCCAATATCAA 3´ Reverse: 5´ CGCGCTTGCCTTTCAGGTCTG 3´	224 bp	Adabi *et al*. (2022)
IS711 B.abortus *IS711 B.abortus*	Forward: 5´ TGCCGATCACTTAAGGGCCTTCAT 3´ Reverse: 5´ GACGAACGGAATTTTTCCAATCCC 3´	498 bp	Garshasbi *et al*. (2014)
IS711 B. melitensis *IS711 B. melitensis*	Forward: 5´ TGCCGATCACTTAAGGGCCTTCAT 3´ Reverse: 5´ AAATCGCGT CTTTGCTGGTCTGA 3´	731 bp	Garshasbi *et al*. (2014)

Table 2: *Brucella* infection in milk samples in different animal species and diagnostic methods

Type of animals	No. of sample	MRT-positive	PCR-positive		
			Brucella genus	*B. melitensis*	*B. abortus*
Sheep	450 (61%)	42 (9.33%)	30 (83.33%)	25 (69.44%)	5 (13.88%)
Goats	190 (25.7%)	4 (2.10%)	6 (16.66%)	5 (13.88%)	1 (2.77%)
Cattle	98 (13.3%)	0	0	0	0
Overall	738 (100%)	46 (6.23%)	36 (78.26%)	30 (83.33%)	6 (16.66%)

USA) with the following steps: primary denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 90 s, primer annealing at 64°C for 1 min, primer extension at 72°C for 1 min and a final extension cycle at 72°C for 5 min. The products were subjected to electrophoresis on a 1.5% agarose gel. In all reactions, standard strains of *B. abortus* ATCC 23455 and *B. melitensis* ATCC 23457 (Razi Vaccine and Serum Research Institute) were used as positive controls and physiological serum was used as a negative control.

Results

MRT

In the MRT examination, 46 samples (6.23%, 95% CI: 2.83-9.63%) showed a positive result. No MRT positive result was detected in the bovine samples. Meanwhile, 9.33% and 2.10% of sheep and goats, respectively, were MRT positive (Table 2).

Molecular biology

All MRT positive samples were tested for the presence of the genus *Brucella* using primers B4 and B5. Of the 46 positive samples, 36 (78.26%) were positive for *BCSP31* (Fig. 2). Of the 36 samples confirmed by *BCSP31* PCR, 30/36 (83.33%) were identified as *B. melitensis* (Fig. 3). Furthermore, amplicons with a molecular size of 498 bp were observed in six samples (16.66%) (Table 2) (Fig. 4).

Discussion

MRT is a highly functional method for identifying infected dairy animals and assessing brucellosis-free herds (Al-Afifi *et al*., 2022). As a cost-effective and easy-to-perform solution, MRT can rapidly screen large populations by detecting IgA and IgM antibodies bound to fat globules (Cadmus *et al*., 2008). Although the test is highly sensitive, it may fail to detect a small number of infected animals within a large herd, based on the milk/antigen ratio in bulk milk samples. Our results showed that 6.23% of the samples were positive by MRT. This prevalence is lower than the 23% reported in eastern Iran using serological techniques (ZareBidaki *et*

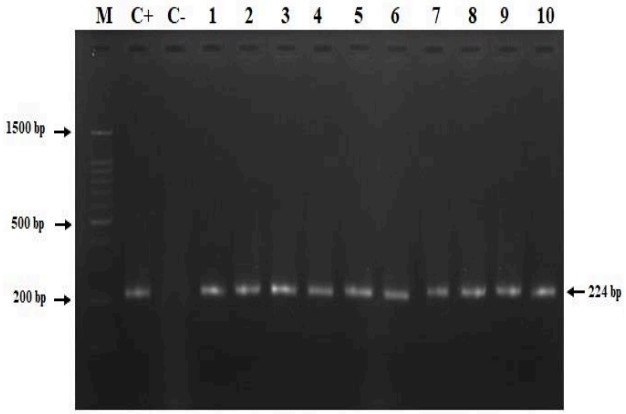

Fig. 2: PCR products electrophoresis to identify the genus *Brucella*. Lane M: 100-bp marker. Lane C+: Positive control (*B. melitensis* standard strain), Lane C-: Negative control (physiological serum), and Lanes 1-10: Positive samples

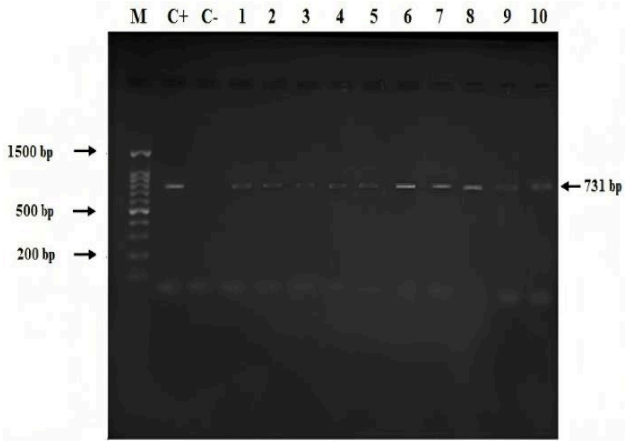

Fig. 3: PCR products electrophoresis to identify *Brucella melitensis*. Lane M: 100-bp marker. Lane C+: Positive control (*B. melitensis* standard strain), Lane C-: Negative control (physiological serum), and Lanes 1-10: Studied samples

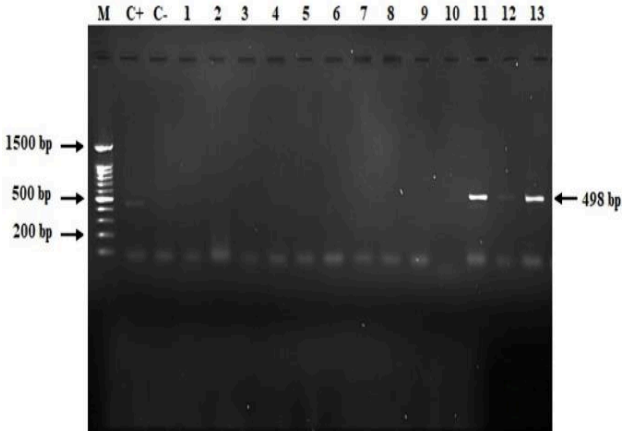

Fig. 4: The PCR products electrophoresis to identify *Brucella abortus*. Lane M: 100-bp marker. Lane C+: Positive control (*B. abortus* standard strain), Lane C-: Negative control (physiological serum), and Lanes 1-13: Studied samples

al., 2022), but higher than the 1.7% found in a meta-analysis of animals in China (Zhou *et al*., 2020). Other studies in Iran have reported different prevalence rates, for example, 3.5% in sheep and 2.7% in goats in southeastern Iran (Sharifi *et al*., 2014) and between 0.85% and 23.3% in countries of the Middle East (Bahmani *et al*., 2022). In addition, an outbreak of brucellosis in small ruminants was reported to be 5.87-18.8% in Egypt, 0.1-1.7% in Pakistan, 2-4% in Yemen, 5.3-10.7% in the United Arab Emirates, 22.2-45.4% in Jordan, 15% reported in Iraq, and 15.6-3.9% in Saudi Arabia (Mustafa *et al*., 2011; Mohammed *et al*., 2013; Abd El-Rahim *et al*., 2014; Bahmani *et al*., 2022).

According to a study by Al-Afifi *et al*. (2022) in Yemen, MRT prevalence rates of brucellosis in sheep and goats were 2.6% and 2%, respectively. The evaluation of other studies using the same method revealed similarities (Al-Afifi *et al*., 2022; Aliyev *et al*., 2022) and differences (Aggad *et al*., 2006; Nofal *et al*., 2017; Béjaoui *et al*., 2022) between our results and those of previous studies. In a previous report by Gharekhani *et al*. (2021) no *Brucella* infection was detected in bulk tank milk samples from dairy farms.

The combination of serological and molecular techniques used in our study provides a more comprehensive detection approach. Molecular confirmation using PCR showed that 78.26% of MRT-positive samples contained the *BCSP31* gene of which 83.33% were identified *B. melitensis*. These findings align with previous research, highlighting a significant prevalence of *Brucella* in small ruminants across the Middle East and North Africa.

Our results, compared to the national average and previous reports, suggested that the prevalence of *Brucella* species was lower in animals in the Famenin region. Furthermore, our results showed that ten samples (21.74%) had positive MRT results, but their PCR test results were negative. These results are similar to those of Garshasbi *et al*. (2014) and Adabi *et al*. (2021) who reported molecular negative results for seropositive samples, which could be due to the cross-reactional reaction of other bacteria possessing similar *Brucella* antigens, frequent exposure to *Brucella* antigens, and insufficient bacterial DNA in the milk.

According to our findings, MRT can be utilized for testing individual and pooled milk samples, making it an inexpensive preliminary test for dairy herds in combination with other tests. The discrepancies between MRT and molecular results highlight the importance of using multiple diagnostic methods to improve accuracy. This combined approach is crucial for the early detection, effective control, and prevention of brucellosis, thereby protecting public health and improving livestock productivity. The presence of brucellosis in the region highlights the importance of improving hygiene practices in livestock farming and the need for regular monitoring using both serological and molecular diagnostic methods.

In summary, this study provides important insights into the prevalence of *Brucella* spp. in lactating livestock in the Famenin region. Regular assessment of the prevalence and distribution of the disease in livestock is crucial for controlling brucellosis. MRT is an effective and simple method for routine screening of milk. However, our study shows that molecular methods are more reliable diagnosis owing to their high specificity and sensitivity, especially in cases where serological tests do not provide clear results.

Acknowledgement

The authors thank the Vice Chancellor for Research and Technology of Hamadan University of Medical Sciences, for financial support of this research (grant No.: 9808216031).

Conflict of interest

The authors declare that they have no competing interests.

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