A comparison of bacteriological culture, serological, and qPCR methods detecting Brucellosis in ewes with a history of abortion

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Abstract

The zoonotic disease Brucellosis is a serious public health and livestock industry concern. In the present study, we used bacteriological culture, Rose Bengal Test (RBT), and quantitative PCR (qPCR) methods to determine the prevalence of brucellosis in serum and milk samples of sheep with a history of abortion. Serum and milk samples were obtained from 100 sheep aged three to five years. To determine the prevalence of brucellosis, a modified RBT was performed on serum samples, *Brucella* was isolated from milk by bacteriological culture, and qPCR was applied to detect bacterial DNA in milk. The prevalence of brucellosis using the modified RBT, bacteriological culture, and qPCR was 32%, 42%, and 44%, respectively. By considering the qPCR as the criterion standard, the modified RBT showed a sensitivity of 95%, a specificity of 100%, an accuracy of 98%, a positive predictive value (PV+) of 100%, and a negative predictive value (PV-) of 97%. The sensitivity, specificity, accuracy, PV+, and PV- for

bacteriological culture were 77%, 100%, 90%, 100%, and 85%, respectively. The agreement between qPCR and modified RBT was 0.959 (95%CI:0.896-1); between qPCR and bacteriological culture was 0.792 (95%CI:0.667-0.897); and between modified RBT and bacteriological culture was 0.831 (95%CI:0.709-0.38). Based on the results, bacterial isolation from sheep's milk is not recommended except in specific cases due to its low sensitivity, time-consuming, and hazardous nature. However, the modified RBT can be used as a routine method because of its cost-effectiveness, higher sensitivity, and accuracy compared to bacterial isolation, qPCR is recommended as the gold standard test for detecting brucellosis in sheep milk, especially in those with a history of abortion.

Abbreviations

RBT: Rose Bengal test

qPCR: quantitative PCR

CI: Confidence Interval

PV+: Positive Predictive Value

PV-: Negative Predictive Value

Introduction

The Brucella genus of non-motile, gram-negative, and intracellular coccobacilli bacteria causes the zoonotic disease Brucellosis [1-3]. Human Malta fever caused by *Brucella*, with more than 500000 cases annually, is the most common contagious disease between humans and farmed species worldwide [4]. The genus *Brucella* with 12 main species, can cause disease in several animal breeds leading to economic loss. For example, abortion, stillbirth and reproductive disorders are common clinical manifestations of brucellosis in sheep [5, 6]. Human infections are mainly caused by *B.melitensis, B.abortus, B.suis and B.canis,* of which *B.melitensis,* with three biovars, is the most contagious [7-9]. *Brucella* infects humans by direct contact with contaminated tissues (placenta, fetus, uterine secretion, etc.) or by consuming unpasteurized dairy products [10, 11]. Contrary to cow's milk used in industrial dairy production, ewe's milk is routinely used for producing raw milk products which increases the risk of contracting Malta fever [12]. In addition, *B. melitensis*, as the major causative agent of brucellosis in ewes, displays higher pathogenicity in humans than *B. abortus* due to its 10000 times less infectious dose [13]. As mentioned, brucellosis significantly impacts on the livestock industry and public health. The control strategies are based on prevention and eradication. Sheep infected by *Brucella* are considered reservoirs in herds. To lessen the risk of disease and subsequent economic losses, the infected sheep must be identified and removed from the herd by the fastest, most cost-effective and least hazardous method. Indeed, the detection of *Brucella* is the fundamental step in any control program. To achieve this goal, laboratory diagnosis could be performed in three diverse areas: 1) direct detection of living bacteria using culture media, 2) indirect diagnosis by serological methods, and 3) rapid diagnosis by molecular assays based upon polymerase chain reaction (PCR) [14].

Bacterial isolation is the most accurate method for Brucellosis detection. However, the chronic stage of the disease is challenged by several limitations, including a long incubation period and

low sensitivity. In addition, appropriate safety precautions need to be implemented for exposed laboratories and workers due to the hazardous nature of the *Brucella* organism classified as a class III pathogen [15]. Although serological methods are recommended for the detection of Brucellosis, they can have false positive and/or false negative results. Indeed, they are either too sensitive causing false positives, or too specific causing false negatives [16]. Additionally, the presence of antibodies in a serum sample does not always indicate an active case of brucellosis like sustained immune responses that form after vaccination [17]. Furthermore, in serological tests several gram-negative bacteria, especially *Salmonella* group N (O: 30), *Escherichia coli* O157:H7, *Yersinia enterocolitica* O:9 and *Vibrio cholerae* O1, can induce antibodies with cross-reactivity and cause false-positive results for brucellosis [18]. Thus, employing two serological tests simultaneously to decrease the number of false positive and false negative results is highly recommended. Regarding the available protocols in Iran, for primary screening, the Rose Bengal Test (RBT) is applied. Then, serum agglutination test (SAT) and 2-mercaptoethanol (2-ME) confirm positive RBT samples. Despite the limited and conflicting information about the RBT [19, 20], this test has been internationally approved for monitoring brucellosis in small ruminants [21]. Rose Bengal can be used as a rapid test for monitoring, but more specific tests are needed to confirm RBT results. SAT is routinely used for confirmation, and titers above a certain threshold are considered active brucellosis. Moreover, 2-ME, combined with SAT, differentiates between the agglutination of IgG and IgM-specific antibodies [22]. Because of the problems raised by the bacteriological culture and immunological methods, developing new diagnostic examinations for directly detecting *Brucella* species in milk has been increasingly under investigation. Recently, quantitative PCR (qPCR), as a well-established method, has been widely used to detect unculturable or slow-growth bacteria in microbial communities. Unfortunately, the number of investigations on *Brucella* detection from ewe's milk by qPCR is relatively limited, so evaluation of the efficiency

of this method for the detection of *Brucella* in ewe's milk is not applicable. However, it seems that a molecular detection method such as qPCR, which targets the specific region of *Brucella* with high sensitivity, could be an appropriate approach for rapid and safe diagnosis of *Brucella* with the lowest rate of false negative and false positive results. This study conducted a real-time PCR assay based on designing an alternative pair of primers to detect *Brucella. spp*. Moreover, the DNA extraction method was proposed to extract DNA of an intracellular pathogen. The present study aimed to: 1) determination of the prevalence of Brucellosis in ewes with a history of abortion by bacterial culturing and qPCR on milk samples and serological methods (Rose Bengal, Wright, 2-ME) on serum samples, 2) comparison of the efficiency of three diagnostic methods (molecular, serological and bacteriological approaches) for identification of the infected ewes, and 3) detection of *Brucella* species circulating in the ewe population by Bruce-ladder multiplex PCR assay.

Results

To ensure the efficiency of the DNA extraction from milk, all samples were evaluated for the integrity of the *GAPDH* (housekeeping gene) in sheep. The 467bp *GAPDH* amplicon detected on a 1% agarose gel (Figure 1) showed the appropriate DNA extraction efficiency.

Comparison of Serological Tests and Culture with the qPCR Test

Out of 100 milk specimens, *Brucella* spp. were isolated from 34 samples (34%), demonstrating the phenotypic and biochemical characteristics of typical *Brucella* species like small and smooth colonies, non-hemolytic small gram-negative coccobacilli, catalase positivity, oxidase positivity and urease positivity. All the isolates grew well in both aerobic and 8-10% CO_2 atmospheres at 37 °C, 4-8 days after incubation. Cultures that did not show any sign of growth until day eight did not grow until the end of 16 days in both atmospheres. Along with the phenotypic assays, the identity of isolated bacteria was confirmed by PCR using genus-specific

primers (Figure 2). Moreover, the species of the *Brucella* strains were defined by the Bruce-ladder multiplex-PCR as well. Forty-two serum samples (42%) were diagnosed as positive by all three serological tests. Among them, all these positive cultures showed positive results in the serological tests. However, eight samples with positive serological results did not show any growth in culture. Using the qPCR assay, genomic elements of *Brucella* spp. were detected in 44 milk samples (44%), 42 of which were serologically positive, and two were serologically negative. All the serological and culture-positive samples were also positive in qPCR (Tables 1 and 2).

The sensitivity, specificity, and positive predictive value (PV+) and negative predictive value (PV-) of serological and culture tests were calculated premised on the qPCR test. The sensitivity, specificity, PV+, and PV- of serological tests compared to qPCR were 95%, 100%, 100%, and 97%, respectively. The mentioned parameters for microbial culture compared to qPCR were 77%, 100%, 100%, and 85%, respectively.

Using the Kappa test, all three methods were evaluated for inter-rater reliability. The agreement between qPCR and modified RBT was 0.959 (95% CI: 0.896-1), between qPCR and culture test was 0.792 (95% CI: 0.667-0.897), and between modified RBT and culture test was 0.831 (95% CI: 0.38-0.709). The positive and negative likelihood ratios (LR) of the diagnostic tests used in this study were also evaluated (Table 3). A positive Rose Bengal or/and culture result is ∞ (infinity) times more likely to originate from an infected animal than from a healthy animal. Only 0.05 times as many animals with brucellosis as animals without the disease will provide a negative Rose Bengal result. An infected animal is 0.23 times more likely to have a negative culture result than a healthy animal.

Table 1.

Prevalence of Brucellosis in sheep with a history of abortion, based on the positive results in each diagnostic test, n (%).

Sample(N)	Microbial culture	RBT	qPCR
100	34 (34%)	42 (42%)	44 (44%)

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Table 2.

Data obtained from Rose Bengal Test, Microbial Culture, and qPCR, including true positive (a), true negative (d), false positive (b), and false negative (c) results.

qPCR	***Brucella* (Modified RBT)**		**Total**	***Brucella* (Microbial culture)**	
	Negative	Positive		Negative	Positive
Positive	(a) = 42	(b) = 2	(a+b) = 44	(a) = 34	(b) = 10
Negative	(c) = 0	(d) = 56	(c+d) = 56	(c) = 0	(d) = 56
Total	(a+c) = 42	(b+d) = 58	n= 100	(a+c) = 34	(b+d) = 66

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Table 3.

Statistical parameters for Modified RBT and Microbial Culture compared to qPCR for diagnosis of brucellosis (95% CI).

Statistic parameter based on qPCR	**Value**	
	Modified RBT	**culture**
Sensitivity	95%	77%
Specificity	100%	100%
Positive Likelihood Ratio	∞	∞
Negative Likelihood Ratio	0/05	0/23
Positive Predictive Value	100%	100%
Negative Predictive Value	97%	85%
Accuracy	98%	90%
Kapa	0.959(95% CI: 0.896 - 1)	0.831(95% CI: 0.709 - 0.38)
	0.792(95% CI: 0.667 - 0.897)	

Bruce-ladder Multiplex PCR

Regarding the capability of The Bruce-Ladder multiplex PCR in identifying the *Brucella* species which are isolated in pure cultures, the test was performed on 32 extracted DNA of *Brucella* bacteria isolated from pure bacterial cultures. By evaluating the patterns of produced fragments on the 1.5% agarose gel patterns represented by Yoldi *et al*., all the isolates (Figure 3) were identified as *B. melitensis* (six amplicons with sizes of 152-bp, 450-bp, 587-bp, 794-bp, 1071-bp, and 1682-bp were multiplied).

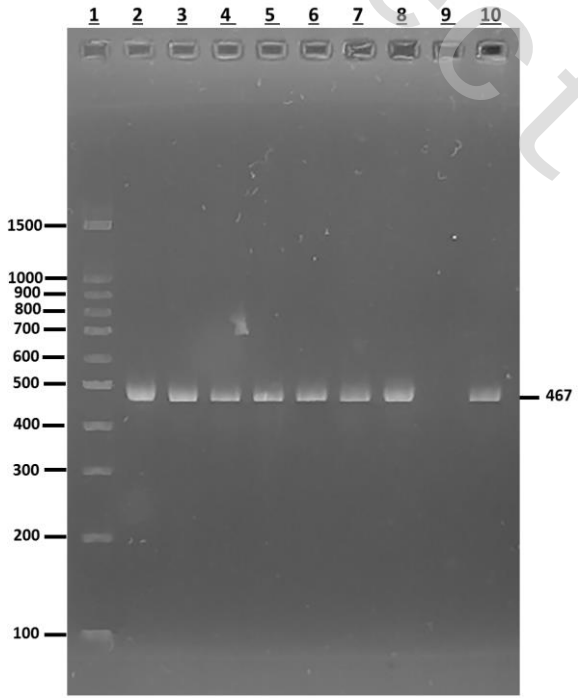

Figure 1.

PCR product of *GAPDH* gene. Lane 1, 100-bp DNA size marker (100-1500 bp); Lane 2-8, *GAPDH* gene; Lane 9, Negative control; Lane 10, Positive control.

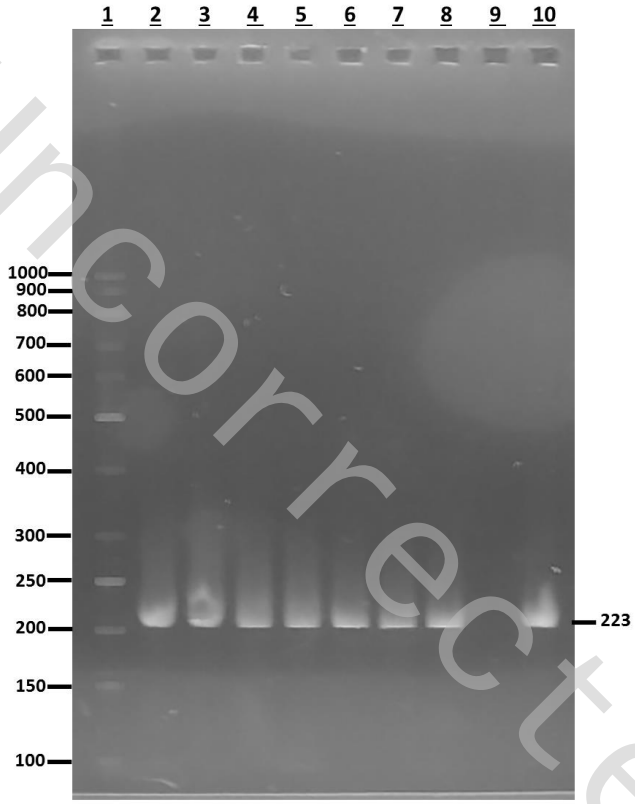

Figure 2.

PCR product of *Brucella* spp. Lane 1, 50-bp DNA size marker (50-1k bp); Lane 2-8, *Brucella spp.*; Lane 9, Negative control; Lane 10, Positive control.

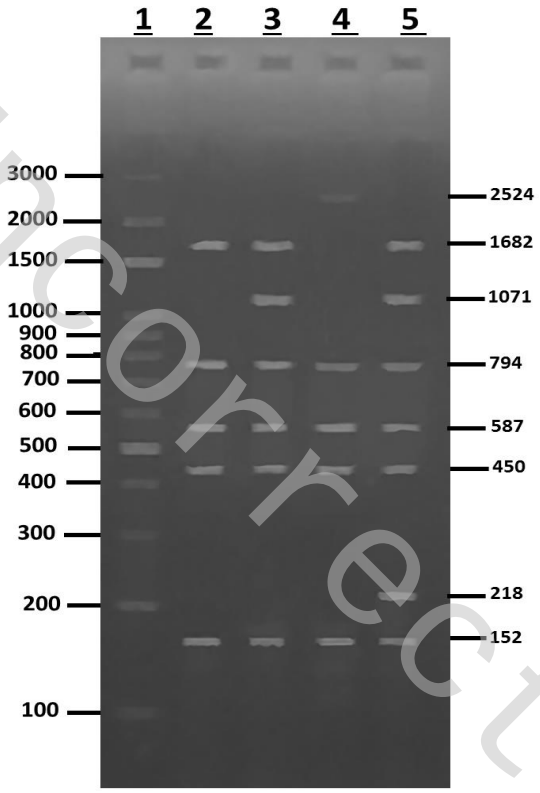

Figure 3.

Differentiation of *B.abortus*, *B. melitensis*, the RB51 and Rev.1 vaccine strains by Bruce-ladder multiplex PCR. Lane 1, 100-bp Plus DNA size marker(100-3k bp).; Lane 2, *B.abortus*; Lane 3, *B.melitensis*; Lane 4, *B.abortus* RB51 vaccine strain; lane 5, *B.melitensis* Rev.1 vaccine strain

Discussion

Brucellosis due to *B. melitensis* is still a major problem for public health and also for sheep herds in several parts of the world, especially in the Middle East and the Mediterranean, since most human cases of brucellosis around the world are infected with this species of *Brucella* spp. *B. melitensis*, the most important zoonotic pathogen between humans and animals among *Brucella* spp., primarily infects sheep as its preferred host and transmits to humans mostly by consuming the milk and dairy products of sheep and goats which are unpasteurized, especially in endemic areas [8, 23-29]. Some clinical symptoms of brucellosis in sheep include abortion, stillbirth, retained placenta, weak lambs, and infertility which cause significant economic loss to the livestock industry [30]. In areas with a high prevalence of brucellosis (more than 5%), using *B. melitensis* Rev. 1 strain vaccine is recommended on a large scale or/and for maiden ewes [28]. In the current study, sheep milk samples were directly subjected to molecular investigation for *Brucella* spp.. The DNA extraction method was applied according to Pokorska *et al* [31], which showed the advantages of low cost, short time, and less required volume of milk compared to many other methods. Studies on the prevalence of brucellosis in sheep have been conducted in Iran and other parts of the world using different methods and conditions of sheep (with a history of abortion or not). In the current research, the prevalence of brucellosis in ewes with a history of abortion was determined by three criteria of assays. In milk culture, 34% of sheep were *Brucella* positive, while serological methods and qPCR on milk samples determined the prevalence of brucellosis at 42% and 44%, respectively. As Al-Talafhah AH *et al*. [32] reported, monitoring the herd status in northern Jordan by RBT showed that 61% of all herds and 14% of sheep in each herd were positive for brucellosis. In another study conducted by Samadi A. *et al*. [33], 86 out of 188 (45.7%) samples of sheep with a history of abortion were positive for brucellosis using PCR. Zhang H *et al*. [34] reported that in the fetal tissues and milk of 120 sheep and cows, the PCR tests for brucellosis were positive for 34 samples

(28%). However, there may be some similarities and differences between the findings of this study and others. Differences in the prevalence of brucellosis can be due to variations in sample types or methods applied in each investigation. In a section of the study conducted by Hamadi *et al*. [17] blood and milk samples of 21 sheep were evaluated for brucellosis using RBT, culture, and PCR methods. Twenty samples were seropositive for the RBT. *Brucella* spp. were isolated from 12 milk samples, while PCR detected *Brucella* spp. in 10 milk samples. 11 PCR-negative samples were positive with the RBT, while a single Rose Bengal-negative sample was positive with the PCR test. In Gupta *et al*.'s [6] study, out of 54 goat samples with a history of abortion, 32 serum samples were positive for SAT. *Brucella* genomic fragments were amplified in 48 milk samples, including 32 serum-positive samples. It was found that the PCR assay, as a controlled experiment, had a specificity of 100% and a sensitivity of 90%. Ilhan *et al*. [2] indicated that by examining the milk samples of sheep with a history of abortion, 8, 24, and 28 samples tested positive with culture, PCR and Milk Ring Test (MRT) methods, respectively. Comparing MRT and PCR tests, 22 positive and 72 negative samples were common in both tests, and a coincidence of 96% was achieved. For the PCR assay, the specificity and sensitivity were estimated at 100% and 81.3%, while for MRT, they were 75% and 75%, respectively. Altun *et al*. [35] evaluated 65 sheep milk samples for antibodies against *Brucella* with indirect ELISA and *Brucella* DNA with qPCR. According to the findings, 6.1% of the samples tested positive in both examinations. Lindahl *et al*. [16] examined blood samples with indirect ELISA and milk samples with qPCR from 570 non-vaccinated cattle. All serum-positive samples were also positive with qPCR, while 8.3% of seronegative cows tested positive for *Brucella* spp. DNA in their milk. In a study performed by Sabrina *et al*. [36] milk samples were obtained from 65 seronegative cows and tested for genomic fragments of *Brucella* with qPCR. Results revealed that 3.08% of cows tested positive for *Brucella* contamination. Zakaria [37] conducted research using 230 blood samples to establish the prevalence of brucellosis through three

different assays: RBT, modified in-house ELISA, and qPCR. The sensitivity and specificity of two serological tests were also calculated using qPCR as a criterion standard. The overall prevalence of brucellosis was estimated at 53.9%, 75.2%, and 79.1% for ELISA, RBT and qPCR, respectively. The sensitivity of RBT was 79.12%, and that of ELISA was 55.49%. In the present study, the qPCR assay identified more positive samples (44 samples) than the culture method (34 samples) which indicates the higher sensitivity of the qPCR method than the microbial culture in detection of brucellosis. Similar results were indicated in studies [37-39] comparing the culture and conventional PCR methods on cow milk, which can be generalized to this study based on the higher sensitivity of the qPCR test than the conventional PCR test. The reason for these results could be linked to the fact that in molecular methods, by targeting the genome of *Brucella*, both live and dead organisms could be detected, while in the culture method, only live organisms could be recognized by growing in a culture medium. Since a small number of *Brucella* organisms can cause the disease, the molecular approach seems more suitable than the culture method for identifying brucellosis in infected animals for control and eradication purposes.

No serological test has been specially defined for *B. melitensis* infection in sheep. It is commonly assumed that the serological tests used for identifying *B. abortus* in cows are sufficient to diagnose *B. melitensis* infection in sheep and other small ruminants such as RBT which is widely used to diagnose brucellosis in sheep while it is mainly designed for *B. abortus*. Standardizing the antigens is a major challenge that affects the sensitivity of the RBT. The antigen standardization conditions that appear suitable for the detection of *B. abortus* in cows are insufficient for *B. melitensis* diagnosis in sheep [20, 40]. Moreover, the RBT has specific limitations, such as anti-complementary activity, the prozone effect that requires heat-inactivated serum [41], and the low sensitivity of the RBT confirmed in culture-proven cases [15, 42]. The Rose Bengal serology test used in this study demonstrated negative results for

two sheep, while the qPCR detected *Brucella* genomic fragments in the milk of these sheep. The results were similar to those reported by Leal-Klevezas *et al.* [38]. In spite of the method recommended by Abdoel T *et al.* [40] to increase the volume of serum used in RBT in order to enhance the sensitivity of the test, the results of the present study are not yet acceptable. This finding alarms and confirms that the sensitivity of the RBT when testing blood samples of sheep requires improvement. However, modifying the antigen used in the RBT by reducing the pH or cell concentration of the antigen may enhance the RBT sensitivity to an acceptable level when using sheep serum.

The qPCR assay proposed in our study, demonstrated advantages over the conventional microbial culture method, including higher speed and greater sensitivity. Moreover, there is no requirement for live *Brucella* organisms in this method which reduces the chance of infection transmission to laboratory staff and increases safety. Finally, it is recommended to use the qPCR method to diagnose or confirm the presence of *B. melitensis* in sheep milk as a stand-alone method or in combination with other methods as a part of control and prevention programs. Although estimating the prevalence of brucellosis was not the main objective of our study, the results revealed that despite vaccination and other control measures over the years, clinical brucellosis still exists in sheep in various parts of the country and is one of the main causes of both sheep abortion and human brucellosis. Since this study was only conducted on a small population of sheep with a history of abortion, further extensive research at the national level is required to target the whole population of traditional and nomadic herds [26] using qPCR techniques alongside other diagnostic methods as a sensitive, accurate, rapid, and easy method. That can prevent the remaining infected sheep from being a false negative source of contamination in the herd.

Conclusion

One of the main measures of the control and prevention program for brucellosis is identifying infected animals. Screening is the first and most important step in test-and-slaughter strategies. The discrepancy between the serological and qPCR methods highlights the need for additional diagnostic strategies to detect serologically false negative animals in screening, control and eradication programs for Brucellosis. However, in countries with limited resources, test-and-slaughter cannot be implemented; identifying infected animals in herds allows farmers to take appropriate protective measures to reduce the spread of the disease.

Materials and Methods

Sampling

A total of 200 milk (n = 100) and blood (n = 100) samples were collected from ewes of different flocks with a history of abortion, aged 3 to 5 years, vaccinated with Rev.1 vaccine at the age of 6 months and had not received any antibiotic or corticosteroid drugs for at least one month before sampling. Subsequent disinfection with 70% alcohol, Blood specimens were taken from the jugular vein using a 5 mL sterile syringe and placed in tubes without anticoagulant. Before collecting milk samples, each teat was washed with warm water and wiped with a disposable towel. Initially, the first squirts of milk were disposed of. Then, about 10 mL of milk was collected from every teat in a sterile 50 mL Falcon tube. To prevent cross-contamination, the gloves were changed after each sampling. After taking the specimens under hygienic conditions, they were kept on ice and transferred to the laboratory within a maximum of three hours. The milk in the falcon tubes was divided into two sterile 15 mL tubes under laboratory conditions, one of the tubes was used right away for microbial culture, while the second tube was stored at a temperature of -80 °C for conducting molecular experiments in the future. It should be noted that all the manipulations of the samples and cultures in the laboratory were performed in a class II biological safety cabinet and national and international guidelines for dealing with *Brucella*-contaminated materials were followed.

Serological Test

Serological tests are part of control and eradication programs for the detection of *B. melitensis* infection in ruminants.

Rose Bengal Test (RBT)

To reduce false negative results, the modified RBT, introduced by Blasco *et al*. [40], was used to increase significant sensitivity without affecting specificity [40, 43]. Briefly, 75 microliters (μL) of serum obtained from the studied ewes' blood were mixed with 25 μL of *Brucella* antigen

(Razi Vaccine & Serum Research Institute, Iran) at room temperature on a flat white ceramic plate and gently shaken for 4 minutes. Any agglutination that appeared during this time was recorded as a positive reaction.

Serum Agglutination Test (SAT) and 2-MercaptoEthanol (2-ME) Test

The Wright and 2-ME tests were applied to confirm the positive results of modified RBT. For the SAT, serum samples were prepared using a solution of sodium phenol chloride with a dilution ratio of 1:80, mixed with an equal volume of *Brucella* antigen (Wright Tube Kit®, Pasteur Institute, Iran) resulting in a 2-fold dilution. After incubating samples for 24 hours at 37°C they were examined for agglutinated particles, and serum titers of 1:80 or higher were considered positive. The 2-ME test was performed for SAT-positive serum samples, with a 1:4 ratio of serum and the 2-ME solution mixed and incubated at 37°C for an hour. Then, a solution of sodium phenol chloride with a dilution range of 1:80 was added, resulting in a 2-fold dilution of the reactions. After incubating for 24 hours at 37°C and resting for one hour at room temperature, the serum samples were examined. A positive result was reported for the 2-ME test when the serum titers were 1:40 or greater.

Microbial Culture and Bacterial Isolation

Samples and *Brucella* strains were cultured in the CITA selective culture medium described by De Miguel *et al*. [44], which is also recommended by the World Organisation for Animal Health (OIE), for the isolation of *brucella* isolates, especially smooth *brucella* species such as *B. melitensis* and *B. abortus*. Briefly, the CITA selective medium consists of blood base agar plates containing 5% sterile sheep serum and supplemented with antimicrobial agents as follows: antifungal agents Amphotericin B (4 mg/liter), Nystatin (100,000 IU/liter) (Solarbio Science & Technology Co., Beijing, China), as well as antibiotics Vancomycin (20 mg/liter), Colistin (7.5 mg/liter), and Nitrofurantoin (10 mg/liter) (Solarbio Science & Technology Co., Beijing, China). Milk specimens were centrifuged at 3000 ×g for 15 minutes at 4°C. Then loopfuls of

both cream and sediment were used for simultaneous inoculation onto two CITA plates. The plates were then incubated in two different atmospheres: aerobic and with 8-10% carbon dioxide (Microbiology Anaerocult c®, Merck, Darmstadt**,** Germany) at 37°C for up to 16 days. The plates were evaluated for bacterial growth every three days starting from day 4, and if no growth was observed after day 16, the culture was reported as a negative result. In the case of bacterial growth observation, a pure culture was prepared for further phenotypic and molecular confirmation. Phenotypic characteristics for confirmation of *Brucella. Spp* such as colonial morphology, bacterial morphology and gram staining, catalase, oxidase and urease activity were recorded.

Molecular tests

In parallel to the microbial culture, the molecular method using DNA extracted from isolated strains was performed for genotypic identification, using genus-specific primers for genus detection and Multiplex Bruce-ladder PCR for diagnosis of *Brucella* species.

DNA Extraction

DNA extraction was performed for the pure cultures using the modified boiling method introduced by Queipo-Ortuño *et al*. [45]. In summary, the bacteria obtained from pure culture were washed twice with Tris-HCL-EDTA buffer and centrifuged at 15000 ×g for 10 minutes. Approximately 600 µL of the top layer of the second centrifugation was removed, and the tube with the remaining material was incubated in a water bath at 100 °C for 10 minutes. After keeping on ice for 10 minutes, the tube was centrifuged at 15000 ×g for 10 minutes. The supernatant was separated and placed at -20 °C for further use. To perform DNA extraction from milk samples, we followed the method previously described by Pokorska *et al.* [31]. In brief, 10 mL of milk collected during sampling was centrifuged at 7000 ×g for 10 minutes at 4°C. The liquid layer on top of the tube along with the fat from the milk were removed, and the

remaining pellet at the bottom of the tube with its supernatant liquid was transferred to a sterile 2 mL tube. The mixture then underwent the process of centrifugation at 5000 ×g for 3 minutes at 4 °C, and the liquid layer on top was removed. The pellet was washed with 1 mL of buffer (15mM Tris-HCl (pH 7.4-7.6), 25mM NaCl, 5mM MgCl2, 15mM Na2HPO4, 2.5mM EDTA, 1% sucrose) by centrifuging at 5000 ×g for 3 minutes at 4 °C, and discarding the supernatant liquid. This step was repeated until the supernatant liquid became clear. Then, 1 ml of lysis buffer (pH 8.8; 6% SDS, 3mM MgCl2, 15mM Tris-HCl, 0.5% DMSO, 6% acetone) was added to the pellet obtained from the preceding step and incubated at 65 °C in a water bath for about 60-90 minutes until the pellet was dissolved entirely. Next, the mixture was cooled at room temperature, and 450 µL of precipitating buffer (2.35M NH4Cl, 1.15M NaCl, 38% ethanol pH 5.0) was added. After Vortexing and centrifuging at 16000 ×g for 8 minutes at 10°C, the liquid on the surface was transferred to a new tube, and 600 µL of 100% isopropanol was added. The tube was then centrifuged at 10000 ×g for 8 minutes, and the remaining liquid on the surface was removed. The DNA pellet obtained was washed twice with 70% ethanol and air-dried. Then, the DNA pellet was dissolved in 100 µL of TE buffer (pH 8.0, 10 mM Tris,1 mM EDTA). Quality and quantity assessment of DNA extracted from milk was beyond the main objectives of this study. However, To confirm the successful DNA extraction process from milk samples, the primers described in the study of Kadivar *et al*. [46] were applied to amplify a 467 bp sequence of a housekeeping gene known as the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (NC_056056.1). Calibrated 1% agarose electrophoresis (Merck, Darmstadt**,** Germany) and Green Viewer safe stain (0.01 v/v) were used to assess the PCR products. A 100-bp DNA ladder (100-1500 bp) (Cat No.YT8503, Yekta Tajhiz Azma, Tehran, Iran) was used as a DNA marker. The sample was stored at -20 °C for further examination if the result was positive.

PCR and Bruce-ladder

The *Brucella spp.* molecular confirmation was conducted on the DNA samples extracted from *Brucella* genus identified positive bacteriologically using genus-specific primers (Metabion International AG, Planegg, Germany) according to the procedures proposed by Richtzen *et al.* [47] and Calibrated 1% agarose electrophoresis was used with a 50-bp DNA size marker (50-1k bp) (DNA ladder III®, Cat No. S-5092-100, Dena Zist Asia, Mashhad, Iran). The characteristics of the primers were applied in the current study is presented in Table 4. Furthermore, considering the capability of the Bruce-ladder multiplex PCR assay in identification of *Brucella* species (*B. melitensis*, *B. abortus*, *B. ovis*, *B. melitensis*, *B. canis*, *B. neotomae*, *B. pennipidialis*, and *B. ceti*) and vaccine strains (*B. abortus* S19 vaccine strain, *B. abortus* RB51 vaccine strain and *B. melitensis* Rev.1 vaccine strain), detection of *Brucella* species was carried out on Brucella genus identified positive, using genus-specific primers and the Bruce-ladder multiplex PCR assay as described by García-Yoldi D *et al.* [48]. In summary, using a thermocycler device (Gene Atlas 322®, Astec Co., Fukuoka, Japan) with a 20-µL mixture containing 10 µL of Taq 2x Master Mix Red (Ampliqon A/S, Odense, Denmark), 4 µL of a primer mixture (Metabion International AG, Planegg, Germany), 1 µL of template DNA, and 5 µL of UltraPure™ DNase/RNase-Free Distilled water, PCR was performed as follows: initial denaturation at 95°C for 7 minutes, followed by a total 25 cycles of 35 seconds of template denaturation at 95°C, 45 seconds of primer annealing at 64°C, and 180 seconds of primer extension at 72°C, with a final extension at 72 °C for 6 minutes. PCR products were analyzed by calibrated 1.5% agarose electrophoresis with Green Viewer safe stain (0.01 v/v) and a 100-bp Plus DNA size marker (100-3k bp) (DNA ladder II®, Cat No. S-5091-100, Dena Zist Asia, Mashhad, Iran). Moreover, *B. melitensis* Rev.1 vaccine strain, *B. abortus* RB-51 vaccine strain (used in the Iranian veterinary organization's vaccination program) and *B. melitensis* strain were used as positive controls.

411 qPCR Assay Design and Setup

412 To detect most variants of *Brucella*, designing primers was performed for the conserved region
413 of the all complete genome sequence of *B. ceti* (NC_022905.1), *B. abortus* (NC_007618.1), *B.*
414 *melitensis* (NC_003317.1), *B. canis* (NC_010103.1), *B. microti* (NC_013119.1), *B. neotomae*
415 (NZ_UIGH01000001.1), *B. ovis* (NC_009505.1), and *B. suis* (NC_004310.3) by beacon
416 designer (version 8.10, Premier Biosoft, USA) (Table 4). Using the Basic Local Alignment
417 Search Tool from the GeneBank database and Snapgene software (version 3.2.1, USA), the *in*
418 *silico* specificity was examined. The qPCR assay was conducted using a 10-µL mixture
419 containing: 5 µL of Real Q Plus 2x Master Mix Green (Ampliqon A/S, Odense, Denmark), 1
420 µL of reverse and forward primers (Metabion International AG, Planegg, Germany), 1 µL of
421 template DNA, and 3 µL of UltraPure™ DNase/RNase-Free Distilled water. Amplification and
422 detection were performed using a real-time device (mic-PCR®, Applied Biomolecular Systems
423 Co., Australia). The process of thermocycling was carried out using the following set of
424 instructions: activation step performed at 95 °C for 15 min. The template was subjected to a
425 total of 35 cycles comprising 30 seconds of denaturing at 95 °C and 30 seconds of annealing at
426 60°C. After completing the annealing step, melting curve analysis was performed within the
427 temperature range of 65°C to 95°C. The baseline and threshold were set using the auto baseline
428 and threshold feature in mic-PCR® Software v2.6.4 (Applied Biomolecular Systems Co.,
429 Australia). Before data analysis, the melting curve (Figure 4) was recorded for each reaction,
430 and by examining these curves, the accuracy of the peak related to the desired DNA fragment
431 and the absence of primer dimers were confirmed. Also, in all qPCR tests performed in our
432 study, if the cycle threshold (Ct) values were 35 or lower, they were considered positive. All
433 samples were tested twice, and if the qPCR results for both times were positive, that sample
434 was reported positive for the presence of *Brucella* spp.

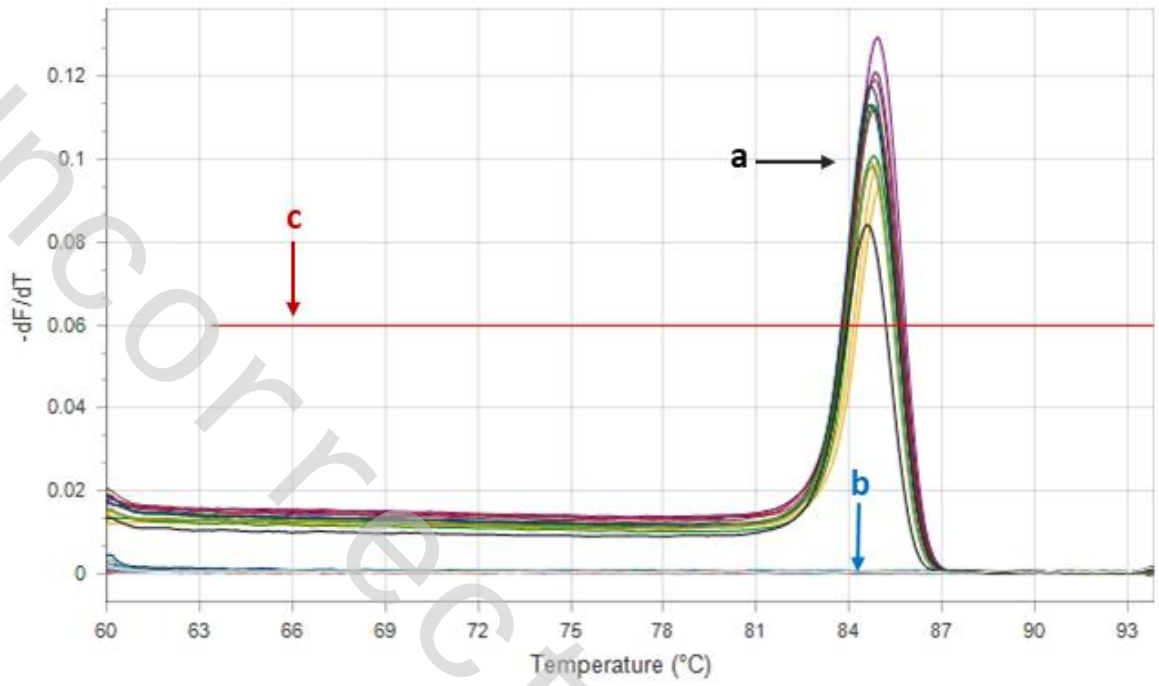

Figure 4.

Melting Curve Analysis for *Brucella spp.* in qPCR test; a, Positive control and positive unknown samples; b, Negative control and negative unknown samples; c, Threshold line

435

436

437 **Statistical Analysis**

438 Contingency 2 × 2 tables were created to determine sensitivity, specificity, PV+, PV-, the LR
439 of a positive test result and the LR of a negative test result for Rose Bengal and bacterial culture
440 tests, where the result of qPCR was considered the criterion standard. The agreement between
441 the tests was evaluated using Cohen's Kappa statistics. According to Landis *et al*. [49], The
442 interpretation of the agreement varied depending on the estimated kappa values. In detail: when
443 the values were between 0 and 0.20, the agreement was considered slight, but for values above
444 0.80, it was deemed almost perfect. When the kappa values ranged from 0.21 to 0.40, the
445 agreement was considered fair, whereas values between 0.41 and 0.60 corresponded to a
446 moderate level of agreement. Similarly, a substantial level of agreement was interpreted for

values ranging between 0.61 and 0.80. The SPSS software version 16.0 was used for carrying out the statistical analysis.

Statement of Animal Rights

This study with grant number 3/57600 was issued ethical approval by the Committee on Research Ethics IR.UM.REC.1401.063, which adheres to the ethical guidelines of research from the School of Veterinary Medicine, Ferdowsi University of Mashhad.

Table 4.

Characteristics of the primers used in the PCR reactions

Primer Pair	Primer Name	Sequence (5′ to 3′)	Amplicon Size (bp)
GAPDH		F:TGGCAAAGTGGACATCGTTG R:TGGCGTGGACAGTGGTCATAAGTC	467
Genus ***Brucella***		F: TGGCTCGGTTGCCAATATCAA R: CGCGCTTGCCTTTCAAGGTCTG	223
qPCR		F: TCCTCGGTCCAGACATAG R: GCGATGATTTATTCCGTATCC[a]	142

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