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Low agreement between serological and molecular tests for the diagnosis of cattle brucellosis

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ABSTRACT

Bovine brucellosis, caused mainly by *Brucella abortus,* is an important cows disease that has created a wide-spread public health problem in humans. Diagnosis primarily relies on serological testing; however, these assays lack sensitivity and, more importantly, specificity. In this study, we tried to compare the performance of serological tests routinely applied in Iran with antigen detection tests. Also, we examined *Brucella* species circulating in cows of Fars province, Iran. In addition, the infection rate of *Yersinia entrocolitica* O9 strain as a probable interfering agent in *Brucella* spp. serological tests were evaluated. Supramammary lymph nodes were sampled from 98 *Brucella* spp. reactor cows of Fars province, Iran, analyzed by bacterial culture and molecular tests, including conventional, multiplex, and real-time PCR. *Brucella* spp. was isolated from 5.1% of cultured samples, while conventional and real-time PCR detected in 15 (15.3%) and 21 (21.4%) samples, respectively. All positive samples were identified as *B. abortus*. Notably, 78.6% of seropositive cows tested Brucella spp. negative by both molecular tests and culture at the time of slaughtering, which showed a high false-positive rate of serological testing. As *Y. enterocolitica* O9 was not detected in any lymph node samples, it could be concluded that immunological cross-reaction with this bacterium was not the reason for the few real-time PCR-positive results among *Brucella* reactor cows. In conclusion, real-time PCR provides valuable information about the Brucella species circulating in the slaughtered cows of each region.

Keywords

Brucella abortus, Yersinia entrocolitica, real-time PCR, serological test

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Abbreviations

RBT: Rose Bengal Agglutination Test
STAT: Standard Tube Agglutination Test
ELISA: Enzyme-Linked Immunosorbent Assay
FPA: the fluorescence polarization assay
CFT: Complement Fixation Test
WOAH: World Organization of Animal Health
2ME: 2-Mercapto-Ethanol
PCR: Polymerase Chain Reaction
B.: *Brucella*
Spp: Species

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Introduction

Bovine brucellosis is most commonly caused by *B. abortus*, less regularly by *B. melitensis,* and rarely by *B. suis,* all zoonosis pathogens of the genus *Brucella* [1]. Transmission to humans occurs primarily through contact with infected reproductive secretions or consumption of infected dairy products [2]. In cattle, *Brucella* organisms tend to localize in the supra-mammary lymph nodes and mammary glands from which they may be shed into milk [3].

The disease must be eradicated from animals to control brucellosis in the human, and this is largely pursued through national tests and slaughter programs in the endemic areas. To identify *Brucella* spp. For infected cows, different methods are available, but because of the limitations of each test, the exact diagnosis of brucellosis in cows is still challenging. Antigen detection tests, such as bacterial culture and PCR, identify the presence of *Brucella* spp. directly. Although bacterial culture is considered the diagnostic gold standard, it is less sensitive, time-consuming, and labor-intensive, and imposes a serious biohazard on laboratory personnel [4, 5]. Antibody detection or serological assays including the Rose Bengal Agglutination Test (RBT), Standard Tube Agglutination Test (STAT), Enzyme-Linked Immunosorbent Assay (ELISA), fluorescence polarization assay (FPA), and Complement Fixation Test (CFT) can be used as screening tests in the control program of brucellosis [4]. However, these tests are hindered by cross-reactivity between *Brucella* species and other Gram-negative bacteria, such as Yersinia enterocolitica O9, Escherichia coli O157, Francisella tularensis, Salmonella urbana, Vibrio cholera, and stenotrophomonas maltophilia [6]. The structural similarity of smooth lipopolysaccharide O-chain between *Brucella* spp. and these bacteria, underlies this problem. For example, Muñoz and colleagues (2005) reported that up to 15% of cattle herds in brucellosis-free regions produced false-positive results in serological tests due to cross-reaction with Yersinia enterocolitica O9 [7]. Consequently, a single serological test is not sufficiently reliable for screening individual animals [4]. To improve accuracy, at least two antigen and/or antibody detection tests are required to confirm the cattle brucellosis [8]. According to diagnostic regime recommended by the World Organization of Animal Health (WOAH), serological tests are applied in Iran to diagnose positive reactor cows. Serum samples from semi-industrialized and industrialized dairy farms are first screened by RBT, with positive sera are tested using STAT, including Wright's test, and 2-Mercapto-Ethanol (2ME) tests. Animals identified as positive reactors are then slaughtered with biohazard precautions. Because the test results drive the slaughter decisions, the sensitivity and the specificity of these tests are critical. So, paying more attention to the test strategies used to identify *Brucella* spp. in farm animals is an important neglected issue.

In the present study, we tried to evaluate the agreement between serological tests and antigen detection tests for diagnosing bovine brucellosis. Specifically, we evaluated how many serologically positive reactor cows could also be confirmed by bacterial culture, conventional PCR, and real-time PCR. An additional objective was to identify the *Brucella* species infecting cows of Fars province, Iran. Consequently, the presence of Yersinia entrocolitica O9 strain in the lymph nodes of cows was determined to be a probable cause of false-positive results in the brucellosis serological tests.

Result

Out of 98 lymph node samples collected from positive reactor cows, conventional PCR detected *Brucella* genus in 15 samples (15.3%) (Figure 1).

The Bruce-ladder multiplex PCR, designed for *Brucella spp* **absence in most positive reactor cows**

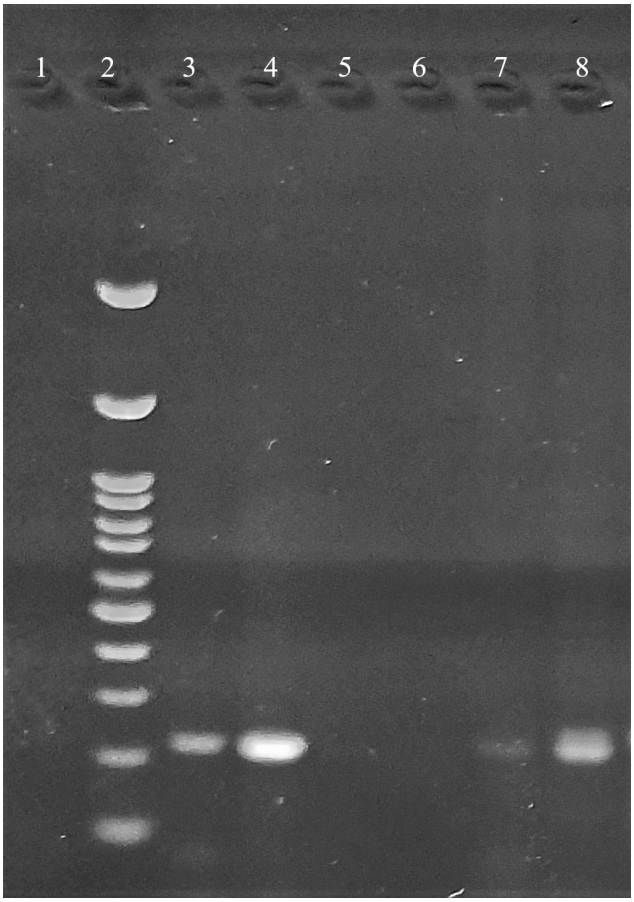

Figure 1.
The gel picture of conventional PCR for Brucella spp. detection. A 223 bp band is obvious in the positive PCR products. Lane 2 shows a 100 bp DNA ladder. Lanes 3 and 4 have different concentrations of positive controls, and lane 1 has no template controls (NTC). Other lanes show samples. An Aliquot of *B. abortus* IRIBA vaccine (Razi, Iran) was used as the positive control.

specie level identification of *Brucella*, did not produce any PCR band from DNA extracted from lymph node tissues. However, when applied to DNA extracted from cultured *Brucella* isolates, the Bruce-ladder PCR was successfully differentiated the species of Brucella isolates (Figure 2).

Real-time PCR analysis identified 21 *Brucella* spp. positive samples (21.4%) out of 98 lymph nodes tested. Melting peak analysis and sequencing of PCR products confirmed all positive samples were *B. abortus* (Figure 3). No statistically significant relationship was observed between real-time PCR-positive samples and the level of 2ME *Brucella* titer.

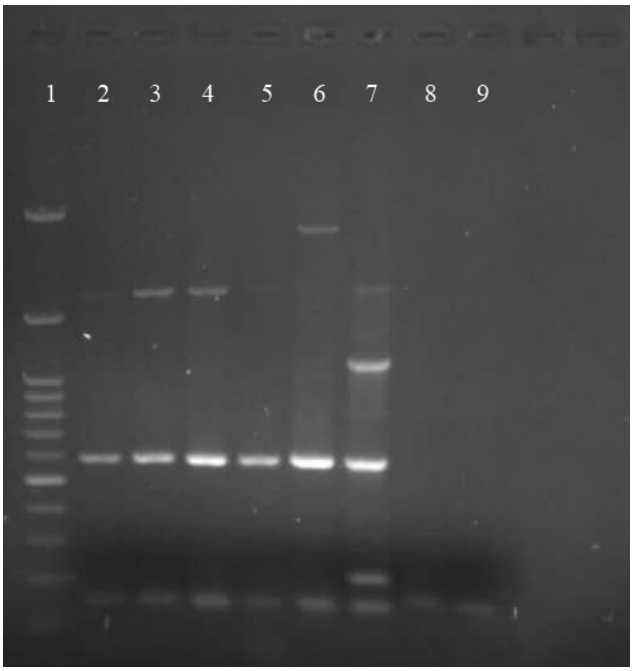

Figure 2.
The gel picture of the Bruce ladder.
Lane 1 was a 100 bp DNA ladder. Lanes 2 to 5 were *B. abortus* from cultured bacterial colonies indicated by 152, 587, and 1682 bp bands. Lane 6 was *B. abortus* IRIBA strain positive control, which was similar to the RB51 strain, showing 152, 587, and 2524 bp bands on the gel, and lane 7 was B. melitensis Rev1 strain positive control, confirmed by 152, 218, 587, 1071, and 1682 bp bands. As 450 and 794 bp bands of the original Bruce ladder did not apply to B. melitensis and *B. abortus* identification, their primers were not used in Bruce ladder PCR.

Brucella spp. was isolated from only 5 samples (5.1%) in bacterial culture, all of which were identified as *B. abortus* using Bruce ladder multiplex PCR.

Given that only 21.4% of positive reactor cows were confirmed positive by real-time PCR, further investigation was conducted to explore the probable reason. As Y. entrocolitica O9 strain was one of the bacteria that might cause serological cross-reactions with *B. abortus* (CSFPH 2018), the prevalence of this strain was evaluated in the lymph node samples. Although 20 lymph node samples tested positive for Y. entrocolitica, none were identified as an O9 serotype.

Discussion

The study demonstrated that among 98 lymph node samples collected from positive reactor cows, *Brucella* spp. was detected only in 15 (15.3%) and 21 (21.4%) samples by conventional PCR and real-time PCR, respectively. In a similar study, O'Leary and colleagues (2006) applied conventional and real-time PCR to different samples from serologically *Brucella* spp. positive cows, slaughtered under Ireland's eradication program. They reported *B. abortus* detected in 3 (14.2%) and 4 (19%) out of 21 supra-mammary lymph nodes samples using conventional and real-time PCR, respectively [9], which these results are consistent to our findings. Also, in another study, Tiwari and colleagues (2014) reported that from 132 STAT-positive

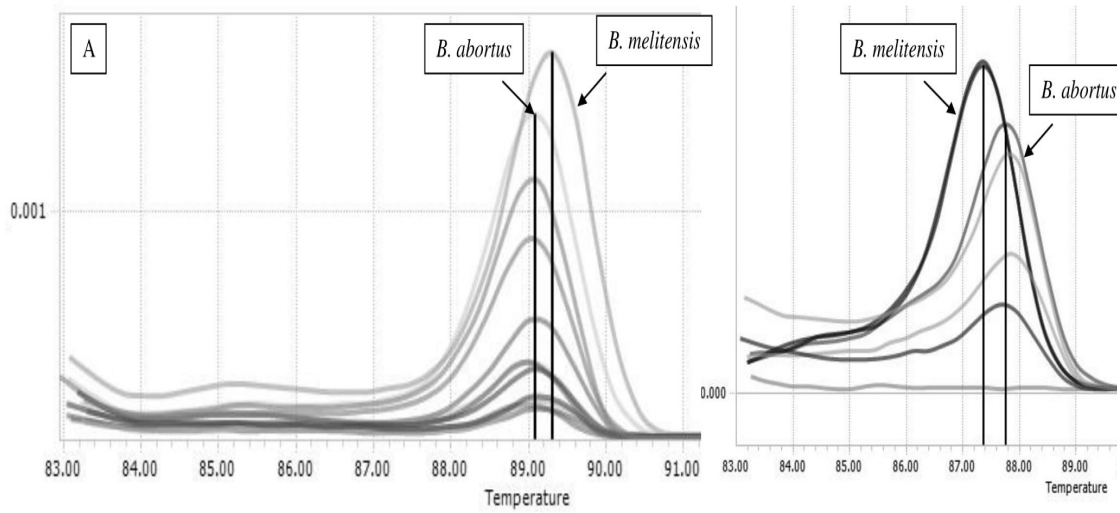

Figure 3.
A) Melting peak analysis of *B. abortus* specific (A) and B. melitensis specific (B) real-time PCR.
The indicator lines show the melting peaks of *B. abortus* IRIBA strain and B. melitensis Rev1 strain positive controls. The graphs show that all of the samples have melting peaks similar to that of *B. abortus* (A) and none of them were located under B. melitensis melting peak (B).

serum samples, only 14 sera (10.6%) were positive by real-time PCR with B4-B5 primers, the same primers used in our conventional PCR assay [10]. A probable reason for the low percentage of PCR-positive results in Tiwari`s study may be partly explained by the type of biological sample, as both our and O'Leary`s studies were conducted on the lymph nodes, whereas Tiwari's was based on serum.

O'Leary and colleagues (2006) also compared *Brucella* spp. detection rate by conventional and real-time PCR across different sample types, including milk, blood, and lymph node. They sampled from both supramammary and retropharyngeal lymph nodes and concluded that the supramammary lymph node is the most reliable tissue for PCR detection of *Brucella* spp. [9]. Their conclusion served as the basis for selecting the sample tissue, and the supramammary lymph nodes were sampled in this research. Nevertheless, according to the tropism of *Brecella* spp. [11], other organs rich in phagocytes, such as the spleen, and organs of the genital system (such as the uterus), could also be suitable sample types for *Brucella* spp. detection.

The real-time PCR result of this study showed that 78.6% of reactor cows were *Brucella* spp. negative at the time of slaughtering. Given that the supramammary lymph node is considered one of the best reservoirs for *Brucella* detection in cows [9], these results suggest that a substantial proportion of reactor cows may be free of infection and are therefore unlikely to shed *Brucella* spp. in milk. These cases were those that *Brucella* spp. bacteria do not remain as an active infection in them. However, their antibody is still detectable by serological tests or individuals never infected with *Brucella* spp. but exposed to other bacteria that immunologically cross-react with *Brucella* spp. Several organisms are known to cause serological cross-reactions with *Brucella,* including Yersinia enterocolitica O9 strain, Escherichia coli O157, Francisella tularensis, Salmonella urbana, Vibrio cholera, and stenotrophomonas maltophilia [6]. As Y. enterocolitica O9 strain was not detected in any lymph node samples, it could be concluded that immunological cross-reaction with this bacterium was not the reason for the few real-time PCR-positive results among *Brucella* reactor cows.

In this study, the sensitivity of conventional and real-time PCR tests was more than that of *Brucella* spp. culture. This observation has been inconsistently reported in the literature. In some studies, PCR sensitivity has been reported more than that of *Brucella* spp. culture method [12, 13]. Hamdy and Amin (2002) compared the sensitivity of PCR and culture methods on bovine milk samples and reported that the PCR sensitivity was greater than that of *Brucella* spp. Culture [13]. whereas in in another study, they reported culture method to outperform PCR [14]. Also, some researchers reported similar results [9]. This study uses Farrell's medium, the most widely used *Brucella* spp. A selective medium was used for the culture prepared by adding six antibiotics to a basal medium. Because some strains of *B. abortus* and B. melitensis may be inhibited by nalidixic acid and bacitracin, two antibiotics in the supplement, the use of this medium may reduce the culture method sensitivity and explain the fewer positive samples of bacterial culture than those of PCR methods.

Molecular characterization in this study identified *B. abortus* as the predominant species infecting cattle in Fars province, Iran. This finding was by multiplex and real-time PCR, and further confirmed by sequencing. Human brucellosis caused by B. melitensis is more severe than the disease caused by *B. abortus* [14], and in terms of public health, B. melitensis is considered a more important zoonosis pathogen. Similar to this study, there are many reports that only isolated *B. abortus* from cow samples from Turkey [15], Pakistan [16], Ireland [9], and Uganda [17], but also there are some studies that isolated B. melitensis in addition to *B. abortus* from cows [18]. The most similar study to ours was performed by Sharifiyazdi et al. (2010), who isolated 17 *Brucella* spp. from 95 positive reactor cows in the same province; of which only one was *B. melitensis,* and the others were *B. abortus* [19]. By comparing these results, it could be concluded that the *Brucella* species infecting cows of this region have not changed from 14 years ago, and cows in Fars province are not the source of human B. melitensis infections.

Finally, it could be concluded that the current serological test combination was conducted in Iran according to WOAH to diagnose the *Brucella* spp. antigen detection tests do not confirm infected cows. We have to know that the lack of specificity in the test regime could waste many healthy cows, limiting the government's potential to widen the brucellosis eradication program to all of the farm animal population, including non-industrialized native cows and sheep. Although real-time PCR is not currently feasible as a routine diagnostic tool directly on serum sample, this test could provide valuable information about the *Brucella* species circulating in the slaughtered cows of each region.

Materials and Methods

Cattle Herds and Sampling

Serum sampling was performed on semi-industrialized and industrialized dairy farms across all regions of Fars province, Iran, under the national brucellosis control program. All cows were lactating Holstein or crossbreeds, raised in the intensive farms. They

had been vaccinated against brucellosis following to the Iranian Veterinary Organization (IVO) guidelines [20] using a vaccine (RVSRI, Iran) containing the IRIBA strain of *B. abortus*. Infected cows were diagnosed using serological assays, including RBT, Wright's agglutination tests, 2-ME agglutination tests, performed in IVO laboratories according to the WOAH guidelines [4]. RBT was applied as the initial screening test, with RBT-Positive sera further evaluated by Wright's agglutination tests and 2-ME agglutination tests. Interpretation of results took into account cow age, vaccination history, and the prior brucellosis condition of the sampled farm. The positive RBT cows would be divided into positive reactors (≥ 4/80 Wright and 4/40 2-ME titers), doubtful, and negative (≤ 1/20 in both tests) cases. Brucellosis cases were retested 3 to 4 weeks later to confirm their status [20].

In this study, supramammary lymph nodes were sampled from 98 serologically *Brucella* spp. positive cows from 20 farms in Fars province, Iran. Lymph nodes were obtained after slaughtering under the national brucellosis control program. Samples were transferred to the laboratory in cool boxes and stored at -20 °C until use.

Bacterial culture

One of the supramammary lymph nodes was transferred to the laboratory of the Department of Brucellosis, Razi Vaccine and Serum Research Institute (RVSRI), Iran, the only nationally authorized laboratory for *Brucella* spp. culture from animal samples. Samples were cultured on *Brucella*-specific agar enhanced with 7% defibrinated sheep blood and *Brucella* supplement (Oxoid, UK). The supplement contained the following quantities of antibiotics for 1 liter of agar: polymyxin B sulfate (5000 IU); bacitracin (25,000 IU); natamycin (50 mg); nalidixic acid (5 mg); nystatin (100,000 IU); vancomycin (20 mg). Plates were incubated at 37°C in 10% CO2 for 21 days. Colonies were identified as *Brucella* spp. based on morphology, serology, and conventional biochemical assays (catalase, oxidase, and urease tests).

DNA extraction

The second supramammary lymph node was used for DNA extraction. Firstly, an emulsion was prepared using a pestle and mortar from 100 µg ground section of each lymph node. Nucleic acid was extracted using a bacterial DNA isolation kit (Denazist Asia, Iran) from emulsion samples according to the manufacturer`s instructions. Some of the extracted DNA was electrophoresed on a 1% agarose gel to check the integrity and purity and the quantity of extracted DNA were determine using a Nanodrop (Bioteck, USA) .

Conventional PCR of Brucella spp.

To detect the *Brucella* genus, a PCR test was conducted in all DNA samples using the following primers: B4: `5-TGGCTCGGTTGCCAATATCAA-`3 and B5: `5-CGCGCTTGCCTTTCAGGTCTG-`3 [21]. A total volume of 25 µl consisted of 1 µl b4 primer (10µM), 1 µl b5 primer (10µM), 12.5 µl Red master mix (Ampliqon, Denmark), 5.5 µl molecular grade water, and 5 µl template DNA. A thermal cycler (BioIntelectica, Canada) was used to run the following PCR program: 5 min at 95 °C as initial denaturation, and 35 cycles of 95 °C 1 min, 63 °C 30 sec, and 72 °C 1 min, followed by 72 °C 10 min.

Multiplex PCR of Brucella spp.

Species-level identification of *Brucella* was performed using the Bruce ladder multiplex PCR. This assay combines eight primer pairs in a single PCR reaction, and *Brucella* species are identified based on each sample's different PCR bands (ladder) [22]. As the bands created by two primer pairs known as BMEI0535f-BMEI0536r and BMEI1436f- BMEI1435r were similar in *B. abortus* and B. melitensis species (expected *Brucella* species in cow), they were not incorporated in a master mix of multiplex PCR, leaving six primer pairs, as shown in the Table 1.

The thermal program consisted of 95 °C 15 min, 35 cycles of 95 °C 35 sec, 63 °C 45 sec, 72 °C 1 min, and finally 72 °C 10 min. 0.62 µl of each forward and 0.62 µl of each reverse primer (10 µM), 12.5 µl of Tempase master mix (Ampliqon, Denmark), and 2.5 µl of DNA sample were mixed (25 µl total volume).

Real-time PCR of Brucella spp.

Two individual Real-time PCR were performed to identify two species of *Brucella* (*B. abortus* and B. melitensis) in all DNA samples using a high-resolution melting (HRM) program. Each real-time PCR differentiates one species from others by comparing the melting peak of an unknown PCR product versus that of a certified positive PCR product. These tests were designed based on a single nucleotide difference in the glk gene of *B. abortus* and the int-hyp gene of *B. melitensis,* with the nucleotide sequence of other species, which causes a slight difference in melting peaks. Real-time PCR primer pairs specific for *B. abortus* and those specific for B. melitensis were named Boa and Bmel, respectively. Their sequences were:

Boa For: `5-GACCTCTTCGCCACCTATCTGG-`3
Boa Rev: `5- CCTTGTGCGGGGCCTTGTCCT-`3
Bmel For:`5- GAGCGATCTTTACACCCTTGT-`3
Bmel Rev:`5- GGACGGTGTAATAAACCCATTGG-`3 [23].

A common thermal program was run by the Light Cycler 96® instrument (Roche, Germany) as follows: initial denaturation of 95 °C for 10 min, then 95 °C for 10 sec and 60 °C for 50 sec repeated 40 cycles followed by HRM program from 65 °C to 95 °C by 0.2 °C /step ramp rate. Some real-time PCR products were sequenced to ensure the substitution of one nucleotide in the glk gene of *B. abortus*.

PCR tests for the detection of Yersinia entrocolitica O9 strain

Two PCR tests were set up to evaluate the Yersinia entrocolitica strain O9 infected cows. Firstly, a PCR test for the detection of all strains of Yersinia entrocolitica was conducted, and then another PCR test was performed on the positive samples of the first PCR to detect specifically the O9 strain. In the first PCR, 227Fmod: (`5-GTCTGGGCTTTGCTGGTC-`3), and YER2: (`5-ATCTTGGTTATCGCCATTCG-`3) primer pair targeting ompF gene, and in the second PCR, perF: (`5-GACGGGGGCAAAAGTAGT-`3), and perR: (`5-CTATTGGGAACACCTCTGGA-`3) primer pair [24] targeting perosamine synthetase gene were used.

In both PCRs, the same master mix components (unless primers), and a common thermal program were applied. For 20 µl total volume of each Y. entrocolitica PCR test, 1 µl of each related primer (10µM) was added to 10 µl Red master mix (Ampliqon, Denmark), 5 µl PCR grade water and 3 µl extracted DNA. Following thermal program: firstly, 95 °C 5 min as initial denaturation, followed by 40 cycles of 95 °C 20 sec, 60 °C 30 sec, 72 °C 30 sec, and finally, 72 °C 7 min as the final extension was applied to PCR microtubes.

To visualize the bands, the PCR products of conventional and multiplex PCR were electrophoresed in 1.5% agarose gels stained with RedSafe (Intron Biotechnology, Korea). The gel pictures were caught by a gel documentation system.

Statistical analysis

Chi-square ($\chi 2$) tests were used to compare the amount of serological 2ME titer and the presence of *B. abortus* in the lymph node samples.

Figure 4 represents a graphical abstract of the materials and methods section.

Table 1.
Names, sequences, amplicon sizes, and target genes of primer pairs used for multiplex PCR, known as Bruce-ladder.

Primer name	Sequence (`5–`3)	Amplicon size (bp)	Target
BMEI0998f	ATC CTA TTG CCC CGA TAA GG	1,682	Glycosyltransferase, gene wboA
BMEI0997r	GCT TCG CAT TTT CAC TGT AGC		
BMEII0843f	TTT ACA CAG GCA ATC CAG CA	1,071	Outer membrane protein, gene omp31
BMEII0844r	GCG TCC AGT TGT TGT TGA TG		
BMEII0428f	GCC GCT ATT ATG TGG ACT GG	587	Erythritol catabolism, gene eryC (Derythrulose- 1- phosphate dehydrogenase)
BMEII0428r	AAT GAC TTC ACG GTC GTT CG		
BR0953f	GGA ACA CTA CGC CAC CTT GT	1,071	ABC transporter binding protein
BR0953r	GAT GGA GCA AAC GCT GAA G		
BMEI0752f	CAG GCA AAC CCT CAG AAG C	218	Ribosomal protein S12, gene rpsL
BMEI0752r	GAT GTG GTA ACG CAC ACC AA		
BMEII0987f	CGC AGA CAG TGA CCA TCA AA	152	Transcriptional regulator, CRP family
BMEII0987r	GTA TTC AGC CCC CGT TAC CT		

As BMEI0535f-BMEI0536r and BMEI1436f- BMEI1435r did not apply to *B. melitensis* and *B. abortus* identification, these two pairs were deleted from the original Bruce-ladder primers.

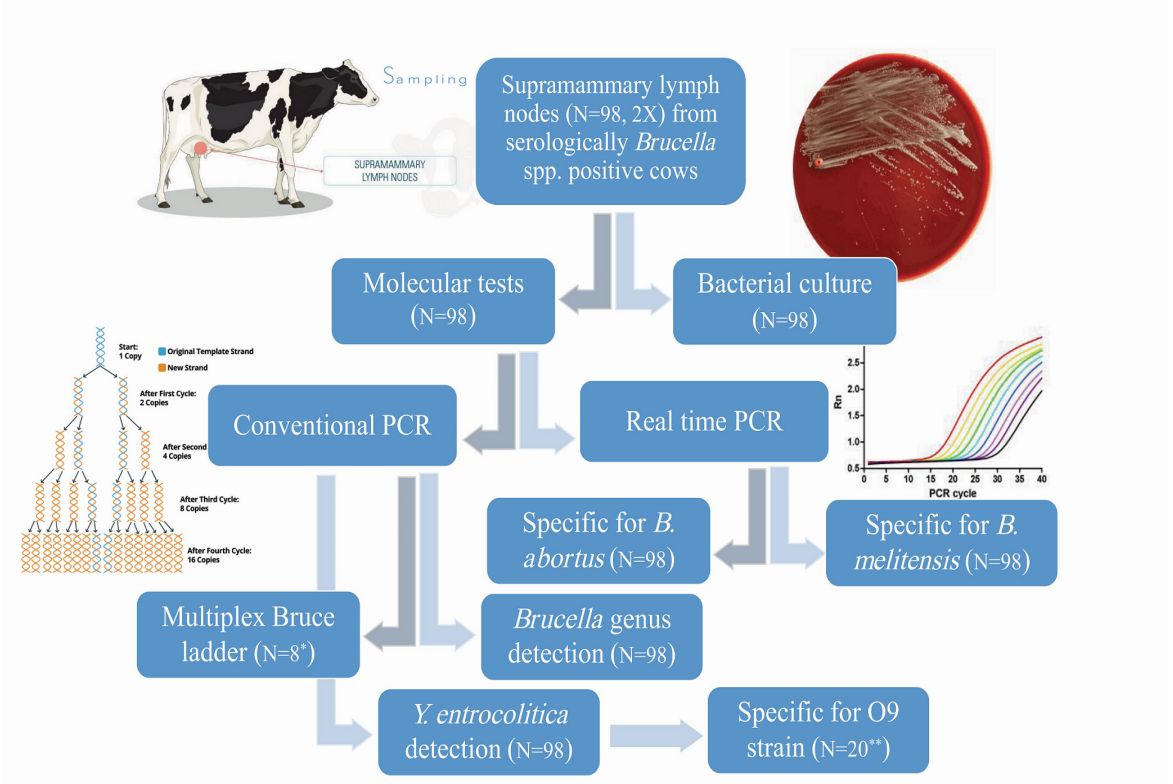

Figure 4.
Graphical abstract.
The diagram shows the sampling and the type of experiments conducted in this study.

Authors' Contributions

All authors contributed to the study conception and design. M.Gh. and M.S.G. performed material preparation, data collection, and analysis. M.Gh. wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The author declares that there is no conflict of interest.

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