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 a significant disease of cows that has created a widespread public health problem in humans. Diagnosis of bovine brucellosis relies on serology, but current serological tests lack sensitivity and, most importantly, specificity. In this study, we tried to compare current bovine brucellosis serological tests in Iran with *Brucella* spp. antigen detection tests. Also, we examined *Brucella* species circulating in cows of Fars province, Iran. Additionally, 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, are used for bacterial culture and molecular tests, including conventional, multiplex, and real-time PCRs. *Brucella* spp. was only isolated from 5.1% of cultured samples and detected in 15 (15.3%) and 21 (21.4%) samples by conventional and real-time PCRs. The species of all *Brucella* spp. positive samples were determined *B. abortus*. Most of the seropositive cows were *Brucella* spp. negative at the time of slaughtering (78.6%) using molecular tests and culture, which showed a high false-positive rate of serological tests for cattle brucellosis. 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 conclusion, real-time PCR could provide valuable information about the *Brucella* species circulating in the slaughtered cows of each region.

Keywords: *Brucella abortus*, *Yersinia entrocolitica*, real-time PCR, serological test

Abbreviations

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

Introduction

Bovine brucellosis is caused frequently by *B. abortus*, less regularly by *B. melitensis*, and scarcely by *B. suis*, all zoonosis *Brucella* species [1]. Contact with infected reproductive secretions or the consumption of infected dairy products is the main transmission route of brucellosis from animals to humans [2]. The supra-mammary lymph nodes and mammary glands of infected cows are important tissues that *Brucella* spp. accumulate in them, and the bacteria could be shed via the secreted milk [3].

The disease must be eradicated from animals to control brucellosis in the human population, mainly 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, like bacterial culture and PCR, detect the presence of *Brucella* spp. bacteria. Although bacterial culture is the gold standard test, it is less sensitive, time-consuming, and labor-intensive, and imposes a serious biohazard on laboratory personnel [4, 5]. Antibody detection or serological tests e. g. Rose Bengal Agglutination Test (RBT), Standard Tube Agglutination Test (STAT), Enzyme-Linked Immunosorbent Assay (ELISA), the fluorescence polarization assay (FPA), and Complement Fixation Test (CFT) can be used as screening tests in the control program of brucellosis [4]. However, the major problem of these tests is cross-reactions between *Brucella* species and some Gram-negative bacteria, such as *Yersinia enterocolitica* O9 strain, *Escherichia coli* O157, *Francisella tularensis*, *Salmonella urbana*, *Vibrio cholera*, and *stenotrophomonas maltophilia* [6]. The great similarity of smooth lipopolysaccharide O-chain between *Brucella* spp. and these Gram-negative bacteria is the cause of cross-reactions. Muñoz and colleagues (2005) reported that up to 15% of cattle herds had false-positive *Brucella* spp. serological tests due to cross-reaction with only *Yersinia enterocolitica* O9 in the brucellosis-free regions [7]. Due to the limitations of each serological method, a single serological test is not sophisticated for screening individual animals [4]. Hence, at least two or more antigen and/or antibody detection tests are required to confirm the cattle brucellosis [8]. According to the World Organization of Animal Health (WOAH), serological tests are applied in Iran to diagnose positive reactor cows. Serum samples collected from (semi-) industrialized

dairy farms are first subjected to RBT, then positive sera are tested using STAT, including Wright, and 2-Mercapto-Ethanol (2ME) tests. Finally, cows assumed as positive reactors are slaughtered with biohazard precautions. As the test results drive the slaughter machine, the sensitivity and the specificity of the different tests used are very important. So, paying more attention to the test strategies used to identify *Brucella* spp. in farm animals is an important neglected issue.

In this study, we tried to evaluate the agreement between serological tests and antigen detection tests of bovine brucellosis. For this purpose, it was determined how many *Brucella* spp. bacterial culture, conventional, and real-time PCRs could also diagnose positive reactor cows. Also, this study's accessory aim was to identify *Brucella* species infecting cows of Fars province. 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.

Results

From 98 lymph node samples of positive reactor cows, conventional PCR detected 15 positive samples (15.3%) for the *Brucella* genus (Figure 2).

Bruce-ladder multiplex PCR, which had been developed to identify Brucella species, did not produce any PCR band from DNA extracted from lymph node tissues. Still,

when DNA extracted from *Brucella* isolates was collected from bacterial culture, the Bruce-ladder PCR could reveal the species of *Brucella* isolates (Figure 3).

Real-time PCR graphs demonstrated 21 *Brucella* spp. positive samples among 98 lymph nodes of positive reactor cows (21.4%). All *Brucella* spp. positive samples were *B. abortus* as indicated by melting peak analysis and sequencing of PCR products (Figure 4). There was no statistical relationship between real-time PCR-positive samples and the level of 2ME *Brucella* titer.

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

After it was found that only 21.4% of positive reactor cows were positive using real-time PCR, it was conducted to find out 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 *Y. entrocolitica* O9 strain in the lymph node samples was evaluated. Although twenty lymph node samples were positive for *Y. entrocolitica*, none of them were identified as an O9 strain.

Discussion

The results of this research showed that from 98 lymph node samples of positive reactor cows, *Brucella* spp. was only detected in 15 (15.3%) and 21 (21.4%) samples by conventional and real-time PCRs. In a similar study, O'Leary and colleagues (2006)

performed conventional and real-time PCRs on different samples from serologically *Brucella* spp. positive cows, which had been diagnosed by serological methods and slaughtered under the Ireland eradication program. *B. abortus* was detected in three (14.2%) and four (19%) out of twenty-one supra-mammary lymph nodes by conventional and real-time PCRs, respectively [9]. These percentages are so close to ours. Also, in another study, Tiwari and colleagues (2014) reported that from 132 STAT-positive serum samples, only 14 sera, equal to 10.6%, were positive by real-time PCR with B4-B5 primers (primers used in our conventional PCR) [10]. A probable reason for the low percentage of PCR-positive results in Tiwari`s study could be that our and O'Leary`s studies were conducted on the lymph nodes rather than serum samples.

O'Leary and colleagues (2006) also compared *Brucella* spp. detection rate by conventional and real-time PCRs in 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 promising tissue for *Brucella* spp. detection by PCR [9]. Their conclusion served as the basis for selecting the sample tissue, and the supramammary lymph nodes were sampled in this research. Although according to the tropism of *Brecella* spp. [11], organs containing large populations of phagocytes, such as the spleen, and organs of the genital system, including the uterus, could 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. As the supramammary lymph node is one of the best reservoir locations of *Brucella* spp. in cows [9], real-time PCR-negative cows might be free of infection and not shed *Brucella* spp. from their milk. These real-time PCR-negative cows 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 those that have not been infected with *Brucella* spp. in their life, but have been contaminated with other bacteria that immunologically cross-react with *Brucella* spp. like *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. There is an inconsistency in other reports. 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]. While the same authors in another study showed that the culture method detected more *Brucella* spp. infected cases than PCR tests [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 sensitivity of the culture method and explain the fewer positive samples of bacterial culture than those of PCR methods.

The *Brucella* species infecting cows of Fars province, Iran, was determined to be *B. abortus* using multiplex and real-time PCRs, additionally confirmed by sequencing. Human brucellosis caused by *B. melitensis* is more severe than the disease caused by *B. abortus* [14]. So, 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 and colleagues (2010), who isolated 17 *Brucella* spp. from 95 positive reactor cows of Fars province; only one of them 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 are not the source of human infections with *B. melitensis* in this regard.

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 cannot currently be used as a routine test for diagnosing *Brucella-infected* cows directly on the 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

The serum sampling was performed from (semi-) industrialized dairy farms from all regions of Fars province under the national brucellosis control program. All of the cows were lactating and were from Holstein or crossbreeds, kept in the intensive farms. They were vaccinated against brucellosis according to the Iranian Veterinary Organization (IVO) guidelines [20] using a brucellosis vaccine (RVSRI, Iran) containing the IRIBA strain of *B. abortus*. The infected cows were diagnosed using serological tests, including RBT, Wright, and 2-ME agglutination tests, in IVO laboratories, according to the WOAH manual [4]. Firstly, the RBT test is used for the screening of brucellosis in bovine serum samples. Positive RBT samples were evaluated with Wright and 2-ME agglutination tests. According to the age and vaccination date of each cow, and the prior brucellosis condition of the sampled farm, the results would be judged. 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-doubtful cows were sampled 3 to 4 weeks later and tested again to be declared [20].

In this study, supramammary lymph nodes were sampled from ninety-eight serologically *Brucella* spp. positive cows from twenty farms in Fars province, Iran. Two lymph nodes were sampled after slaughtering under the national brucellosis control program. Samples were transferred to the laboratory in cool boxes and then frozen at -20 ^{o}C until use.

Bacterial culture

For bacterial culture, one of the 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. The 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). The culture plates were incubated at 37°C with an atmosphere of 10% CO2 for 21 days. Morphological, serological, and conventional biochemical tests, e.g., catalase, oxidase, and urease tests, were used for *Brucella* spp. identification.

DNA extraction

Another 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 was subjected to the Nanodrop (Bioteck, USA) to determine the purity and the quantity of extracted DNA.

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 ^{o}C as initial denaturation, and 35 cycles of 95 ^{o}C 1 min, 63 ^{o}C 30 sec, and 72 ^{o}C 1 min, followed by 72 ^{o}C 10 min as final extension.

Multiplex PCR of *Brucella* spp.

To identify the *Brucella* species of positive cases in conventional PCR, a multiplex PCR known as the Bruce ladder was used. In this test, eight primer pairs are combined

in a single PCR tube, 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, and only six primer pairs were mixed in this study as shown in the Table.

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

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	272	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		

The thermal program consisted of 95 ºC 15 min, 35 cycles of 95 ºC 35 sec, 63 ºC 45 sec, 72 ºC 1 min, and finally 72 ºC 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 PCRs 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 and 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 ºC for 10 min, then 95 ºC for 10 sec and 60 ºC for 50 sec repeated 40 cycles followed by HRM program from 65 ºC to 95 ºC by 0.2 ºC/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 ºC 5 min as initial denaturation, followed by 40 cycles of 95 ºC 20 sec, 60

^{o}C 30 sec, 72 ^{o}C 30 sec, and finally, 72 ^{o}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

χ^2 statistical analysis was used to compare the amount of serological 2ME titer and the presence of *B. abortus* in the lymph node samples.

Figure 1 represents a graphical abstract of the materials and methods section.

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Figures

Figure 1. Graphical abstract.

The diagram shows the sampling and the type of experiments conducted in this study.

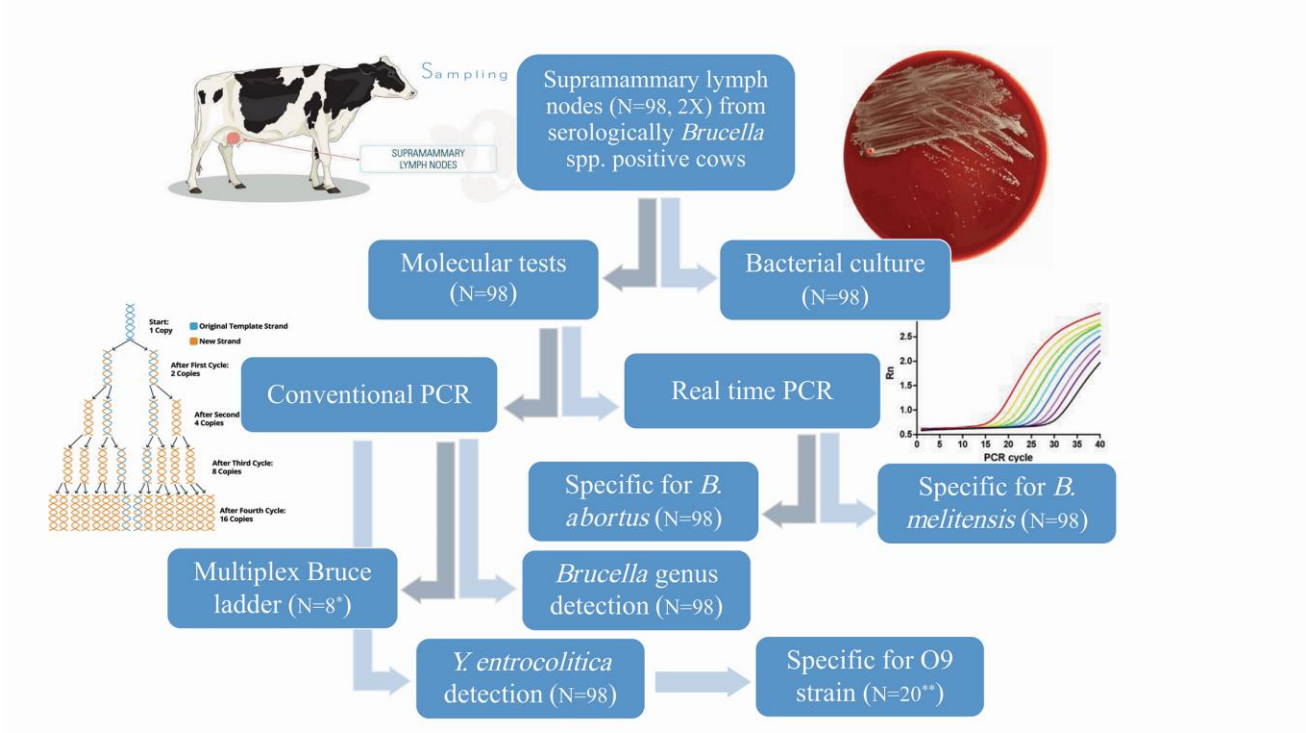


Figure 2. The gel picture of conventional PCR for *Brucella* spp. detection.

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.

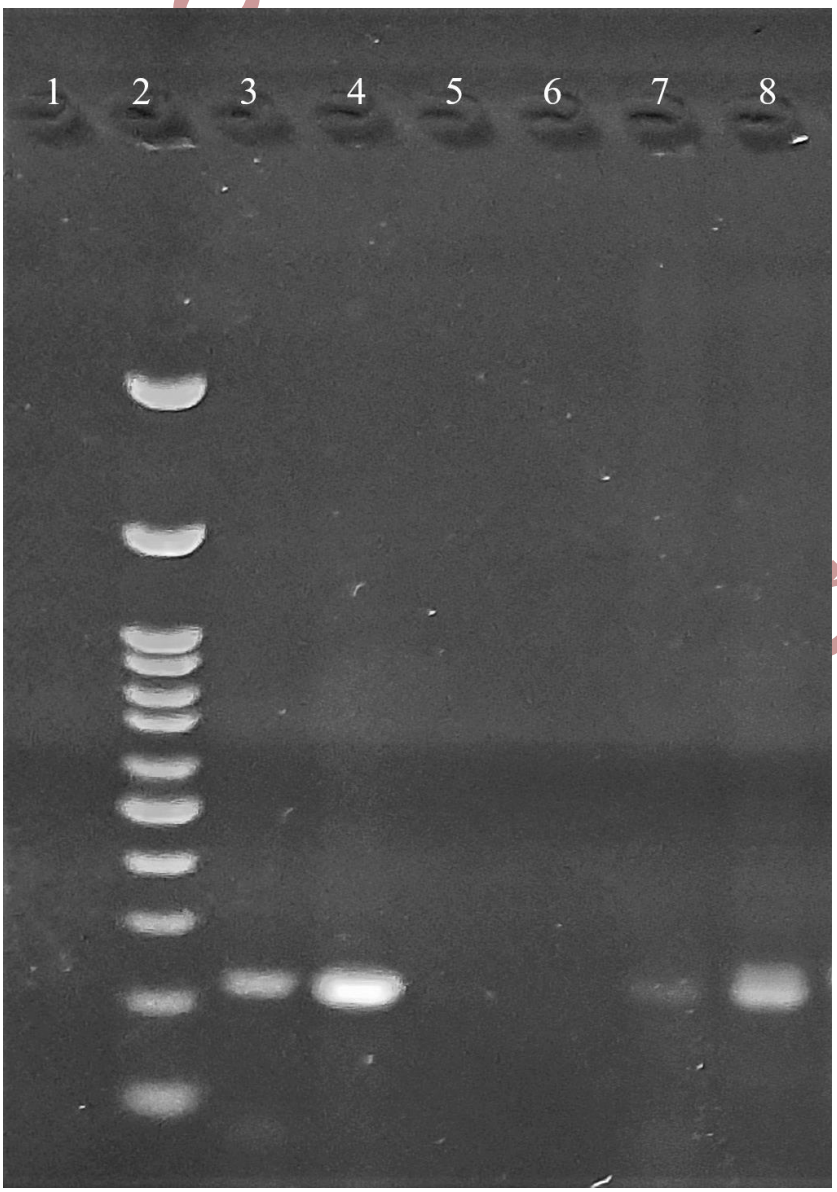


Figure 3. The gel picture of the Bruce ladder.

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.

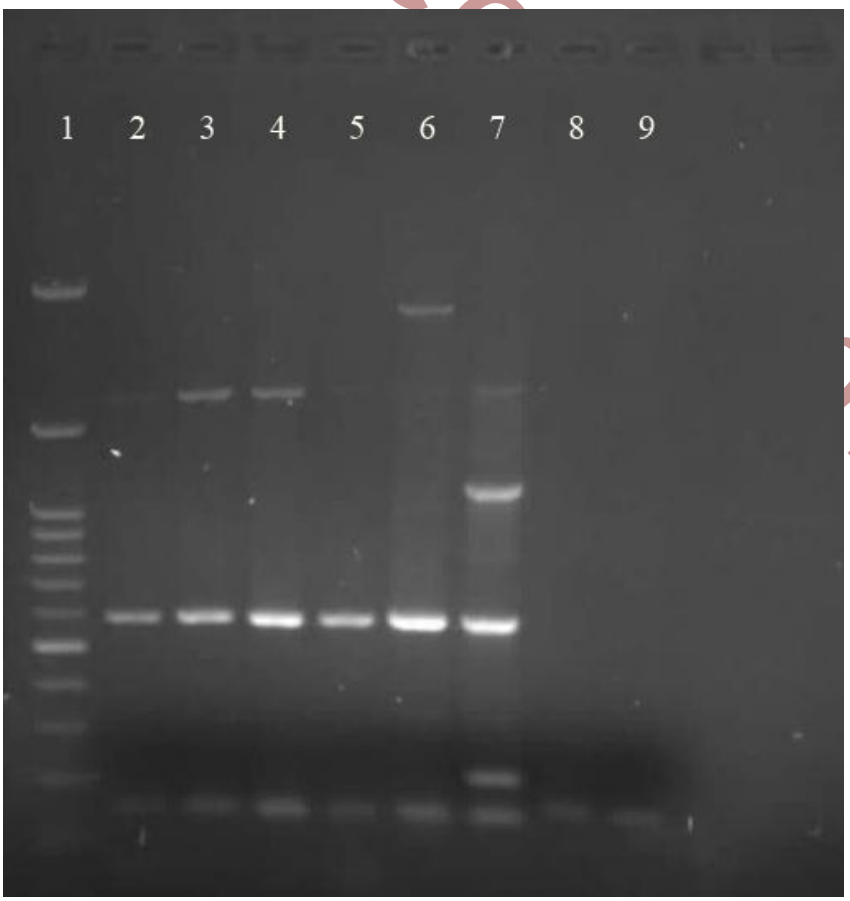
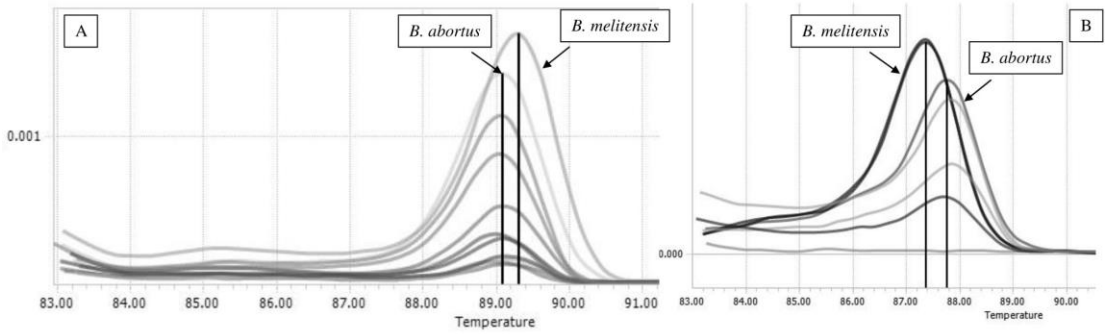


Figure 4. A) Melting peak analysis of *B. abortus* specific (A) and *B. melitensis* specific (B) real-time PCR.

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).



عنوان مقاله: توافق اندک بین آزمایش های مولکلوی و سرولوژی برای تشخیص بروسلوز گاوی

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خلاصه فارسی: بروسلوز گاوی که بیشتر بوسیله *بروسلا آبورتوس* ایجاد می شود، یک بیماری مهم در گاو می باشد. این بیماری مشترک بین انسان و دام بوده و مشکلات زیادی برای سلامت عمومی ایجاد می کند. هم اکنون تشخیص بروسلوز گاوی بر اساس آزمایش های سرولوژی انجام می شود. در این مطالعه، تلاش گردید که آزمایش های سرولوژیک بروسلوز گاوی رایج در ایران را آزمایش های ردیابی انتی ژن مقایسه کنیم. همچنین نرخ آلودگی به *یرسینیا انتروکولیتیکا* O9 به عنوان یک عامل مداخله کننده احتمالی در آزمایش های سرولوژیک گونه های *بروسلا* ارزیابی شد. 96 نمونه از غدد لنفاوی فوق پستانی گاوهای راکتور مثبت در استان فارس اخذ گردید. کشت باکتریایی و آزمایش های مولکولی بر روی این نمونه ها انجام گردید. باکتری *بروسلا* تنها از 8/3 ٪ از نمونه های کشت شده جداسازی گردید و نیز در 15/3٪ و 21/4٪ نمونه ها با روش های PCR معمولی و بی درنگ ردیابی گردید. تمامی بروسلاهای ردیابی شده از گونه *بروسلا آبورتوس* بودند. اینکه در اغلب گاوهای راکتور مثبت، باکتری بروسلا ردیابی نشد نشانگر نرخ بالای مثبت کاذب آزمایش های سرولوژی رایج می باشد که نیاز به بازنگری در آزمایش های مورد استفاده برای شناسائی گاوهای آلوده را نشان می دهد. در نهایت، آزمایش PCR بی درنگ اطلاعات ارزشمندی درباره گونه های *بروسلای* در حال گردش هر ناحیه فراهم می کند.

واژگان کلیدی: *بروسلا آبورتوس*، *یرسینیا انتروکولیتیکا*، تست سرولوژی، PCR بی درنگ