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PCR detection of *Brucella abortus* in cow milk samples collected from Erzurum, Turkey

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Aim: In this study, we designed and used 2 different type-specific polymerase chain reaction (PCR) methods for the detection of *Brucella abortus*.

Materials and methods: There were 2 different primer sets (B4/B5 and AF/AR) selected and used for the amplification of 2 different genes that are present in all biovars of *Brucella* species, including *bcsp*31, encoding 31-kDa immunogenic cell surface proteins, and *omp*25, which is known to be one of the virulence factors encoding outer membrane proteins of 26–23 kDa.

Results: The results showed that 273 (81.7%) and 317 (94.9%) out of 334 milk samples were positive for brucellosis, as detected by either both or one of the primer sets used in the present study, respectively. The detection limit of PCR assays for *Brucella* in milk samples was determined as 5 pg DNA for both of the primer sets.

Conclusion: These results suggest that the use of the specific PCR assay with the primer sets used in the study is a rapid, reliable, and accurate technique in comparison to traditional and conventional methods for the detection and diagnosis of *Brucella* spp. in milk samples.

Kew words: *Brucella abortus*, bovine milk, *bcsp*31-PCR, *omp*25-PCR

1. Introduction

Brucellosis is a zoonotic disease with a considerable impact on human and animal health and remains an important infectious disease, both in Turkey and the rest of the world (1,2). It is well known that this disease causes considerable economic loss by causing a decrease in calving and milk efficiency. It also causes an increase in infertility and poses a great risk to public health when the infection is transmitted to humans (3–5). To date, many studies have been carried out to reduce brucellosis prevalence through standardization of the traditional diagnostic methods and/or implementation of novel methods (6–8), as exact species identification is required to control and reduce brucellosis prevalence. In this regard, much importance has been attached to the studies carried out to keep brucellosis under control in endemic regions (9,10). It is necessary to diagnose new endemic regions and to implement strict eradication programs beyond national borders, because studies have revealed that it is possible there will be a serious increase in the incidence of the disease in the near future, and the problem is growing more severe each year (11). In particular, increase in the surveillance of human brucellosis results from changes in disease epidemiology in some countries (11). In light of this information, several eradication programs have been launched in many countries, but the success of these programs still has not reached the desired level. In these programs, the important steps can be listed as follows: vaccination of the animals open to exposure, carrying out of studies to identify the infected farm animals, and the slaughter of animals and limitation of movement areas in the regions where the infection has been detected.

Diagnosis of brucellosis, which is a cornerstone for eradication programs, is currently dependent on traditional and serologic techniques. The use of a serologic test is a method proposed for indirect diagnosis of the disease; however, today the sensitivity and specificity of serologic tests are not at the desired level because of false positive and/or negative reactions (11,12). This technique is not reliable in early phases of the disease because of

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its low sensitivity, cross-reactions, and failure in making distinction between active and inactive infections due to posttreatment antibody responses (6,13). It is diagnosed through blood culture, which is regarded as the gold standard for laboratory diagnosis of this pathogen, and many difficulties are experienced in routine applications, such as the length of time for the process of diagnosis, difficulties in development due to low amounts of living *Brucella* in the samples, and the risk to the laboratory personnel due to the possibility of aerosol infection (3,6,14,15).

In recent years, studies for the use and development of the low-cost polymerase chain reaction (PCR)-based test techniques proved them to be fast and sensitive in the diagnosis of brucellosis, and it was also shown that they give results in a very short time (less than 4 h) (6,15–17). It is a very important advantage for the technique to be able to detect a few cells of the pathogen or targeted gene copies (16,18). For these reasons, the aim of this study was to develop a one-stage diagnostic PCR test to detect brucellosis in infected and noninfected cow milk samples.

2. Materials and methods

2.1. Milk samples

Milk samples were collected from 334 Eastern Red cows in urban and rural areas of Erzurum, located in the Eastern Anatolia Region of Turkey. These cows had a history of abortion or shared the same barn. In addition, samples for the study were chosen from positive ones with MRT applications. The samples were collected in a sterile cup with a lid and brought to the laboratory as soon as possible, divided into 0.5 mL of sterile 2-mL Eppendorf tubes, and kept frozen until use (15,19).

2.2. Reference strain

Brucella abortus RSK-03026 reference strain, used as a positive control in this study, was provided by the Refik Saydam Hygiene Center Presidency, Ankara, Turkey.

2.3. Isolation of genomic DNA from bacteria

Total genomic DNA was extracted from bacterial samples using the method previously described by Adıgüzel (20).

2.4. Extraction of genomic DNA from milk samples

Total genomic DNA was extracted from milk using a modified method previously described by Arasoğlu (19). Half a milliliter of frozen milk was thawed at room temperature and mixed with 100 µL of NET buffer (50 mM NaCl, 125 mM EDTA, 50 mM Tris-HCl, pH 7.6). To this mixture, 100 µL of 24% sodium dodecyl sulfate (SDS) was added as a denaturing agent. The mixture was cooled after incubation at 80 °C for 10 min. RNase A (75 µg/mL) was added and the mixture was kept at 50 °C for 2 h. Proteinase K (650 µg/mL) was added and the mixture was kept at 50 °C for 1.5 h. Next, a 0.2 volume of 5 M NaCl and 0.1 volume of CTAB-NaCl were added, and the mixture was kept at 65 °C for 10 min. An equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1) was added and shaken for 20 s and centrifuged at 16,000 rpm for 10 min. The supernatant was transferred into a fresh tube and added to a 0.1 volume of CTAB-NaCl, and the mixture was kept at 65 °C for 10 min. All tubes had an equal volume of chloroform and isoamyl alcohol (24:1) added, and they were shaken for 20 s and centrifuged at 16,000 rpm for 15 min. The supernatant was transferred into a fresh tube and precipitated with isopropanol. The pellets were washed with 70% ethanol 3 times, dried, and suspended in 50 µL of TE buffer. The purity of the DNA was determined spectrophotometrically by reading at A_{260} and A_{280} and stored at –20 °C until further use (21).

2.5. Primer design

There were 2 primer sets, including AF/AR, 5'-ATGCGCACTCTTAAGTCTC-3' and 5'-GCCSAGGATGTTGTCCGT-3' (Alpha DNA, Canada) (22) and B4/B5, 5'-TGGCTCGGTTGCCAATATCAA-3' and 5'-CGCGCTTGCCTTTCAGGTCTG-3' (Alpha DNA) (6,19,23,24), selected and used to amplify a target sequence of 490 bp within a gene encoding outer membrane protein *omp*25 (*omp*3a) of 26–23 kDa and a target sequence of 223 bp within the *bcsp*31 gene encoding a 31-kDa *Brucella* spp. antigen, respectively.

2.6. Amplification of *Brucella* DNA by PCR

An amplification reaction mixture was prepared in a volume of 30 µL containing 3 µL of 10× PCR buffer, 0.6 µL of dNTP mixture (10 mM each of dATP, dGTP, dCTP, and dTTP, Sigma-Aldrich Co., USA), 4 µL of each primer (5 µM), 1.2 µL of $MgCl_2$ (25 mM), 0.3 µL of Taq DNA polymerase (5 U/µL, Sigma-Aldrich Co.), 12.9 µL of sterile ddH_2O, and 3 µL of genomic DNA.

The reactions were performed in a thermal cycler (Corbett Research CG1-96, Australia) without mineral oil. PCR master mix (without genomic DNA) and ddH_2O were used as a negative control and DNA from *B. abortus* RSK-03026 was used as a positive control with the sample set. After an initial denaturation at 95 °C for 2 min, the PCR profiles were set as follows: 1 min of denaturation at 94 °C, 1 min of annealing at 59 °C for AF/AR primers and 61°C for B4/B5 primers, and 1 min extension at 72 °C, for a total of 35 cycles, with a final extension at 72 °C for 7 min. The samples were analyzed by electrophoresis in a 2% agarose gel and then stained with ethidium bromide (0.5 µg/mL). The PCR product bands were photographed under ultraviolet light (21).

2.7. Nucleotide sequence analysis

Gel-purified PCR products (Sigma-Aldrich Co.) were sequenced by RefGen Biotechnology (METU Technopolis, Turkey). Sequences were edited with the BioEdit program

(Ibis Biosciences, USA) and compared for similarities with the nucleotide sequences in the National Center for Biotechnology Information library (21).

2.8. Determination of the limit of detection of *B. abortus* in raw milk

The sensitivity of the 2 pairs of primers (AF/AR and B4/B5) was evaluated by using serial dilutions of DNA of *B. abortus*. PCR master mix (without genomic DNA) was used as a negative control and DNA from *B. abortus* RSK-03026 was used as a positive control with the sample set. For this purpose, 2 different studies were carried out. In the first study, the genomic DNA obtained from contaminated milk in different amounts (75 ng, 50 ng, 5 ng, 500 pg, 50 pg, 5 pg, 0.5 pg, and 0.05 pg) of the *B. abortus* RSK-03026 strain were used. In the second study, different amounts (75 ng, 50 ng, 5 ng, 500 pg, 50 pg, 5 pg, 0.5 pg, and 0.05 pg) of the genomic DNA obtained directly from the RSK-03026 strain were used.

3. Results

We collected a total of 334 milk samples from the Erzurum region to use in this study. Samples were collected from the places where the number of cases of brucellosis was high. Cattle that had experienced abortion and were sharing the same stable or using the same pastures were preferred.

The 2 independent PCR assays resulted in the amplification of 223- and 490-bp bands from the targeted *bcsp*31 and *omp*25 genes of the *Brucella abortus* reference strain, respectively. All PCR analyses were repeated twice. The results are given in Figure 1. The accuracy and reliability of PCR data obtained from the *B. abortus* reference strain were confirmed by DNA sequence analysis. In a similar way, the total genomic DNA isolated from the collected milk samples in this study was amplified for *bcsp*31 and *omp*25 genes by PCR as described above (Figures 2–5). The results showed that 273 (82%) and 317 (95%) out of 334 milk samples were found to be positive for brucellosis detected by either both or one of the primer sets used in the present study, respectively.

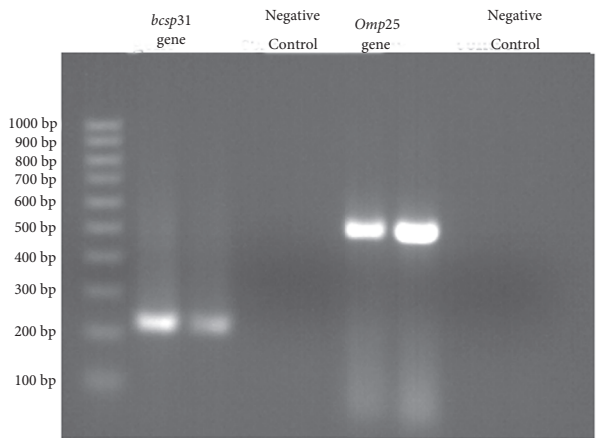

Figure 1. *omp*25 and *bcsp*31 gene amplification results of *B. abortus* strain.

Only 17 milk samples were negative for brucellosis, based on both PCR assays used. The number of brucellosis-positive samples detected either by *bcsp*31-PCR or *omp*25-PCR was 297 (94%) and 294 (93%) out of 334 total milk samples tested, respectively (Table). The detection limit of PCR for pure *Brucella* DNA was determined as 5 pg DNA by each primer set of B4/B5 and AF/AR, as shown in Figures 6 and 7.

4. Discussion

In the present study, we evaluated different methods of extraction of bacterial DNA from bovine milk in order to improve the direct detection of *Brucella* by PCR, and we compared the sensitivity of 2 different PCR methods for the detection of *Brucella* spp. using the same extraction procedure of DNA with conventional PCR.

The extraction of *Brucella* DNA from a whole-milk sample is generally a difficult procedure, as *Brucella* is an intracellular pathogen with a very high affinity to the milk fat layer. Therefore, it is important and better to take the DNA extraction sample for *Brucella* spp. from the upper surface of milk samples under the fatty cream layer. Gram-negative bacterial cell membranes are very sensitive to Tris and EDTA solutions. However, *Brucella* is resistant to nonionic detergents, such as EDTA, but is more sensitive to ionic detergents, such as SDS (25). Thus, high concentrations of Tris, EDTA, and SDS, with increased incubation temperature, were used in our study. The present study included a phenol-chloroform step at the end of the extraction protocol and it had a protein impurity reducing effect on total genomic DNA, which conflicts with some of previous reports (25,26) and confirms the findings of Matrone et al. (27). Therefore, these data indicate that the modified DNA extraction protocol used in our study can also be applied for PCR detection of many other bacterial pathogens in milk.

The detection limit of both primer sets (B4/B5 and AF/AR) for *Brucella* in milk samples was determined as 5 pg pure genomic DNA. Our results demonstrate agreement with the results of Navarro et al. (24), but oppose the results of other studies in the literature (9,23,28). This conflict may be explained by different PCR programs, sample type and preparation, storage conditions, and DNA extraction procedures. This is the first study using the primers set B4/B5 for the detection of *Brucella* spp. from dairy products; all other studies with this primer set concentrated particularly on the reference strains and clinical samples such as blood and serum (16,25,29–31).

The prevalence of *Brucella* in milk samples were determined to be as high as 95% (317 of 334 samples) for

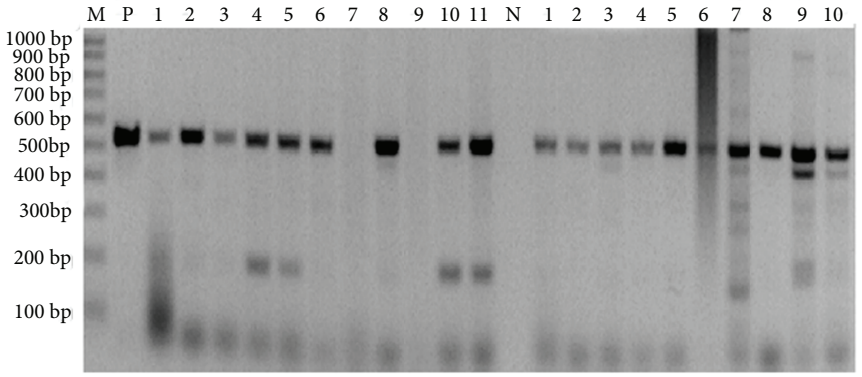

Figure 2. *omp*25 PCR amplification results of milk samples collected from Yukarı Danişment and Kırkgöze villages. M: marker, P: positive control, 1–11 Yukarı Danişment village milk samples; 1–10 Kırkgöze village milk samples.

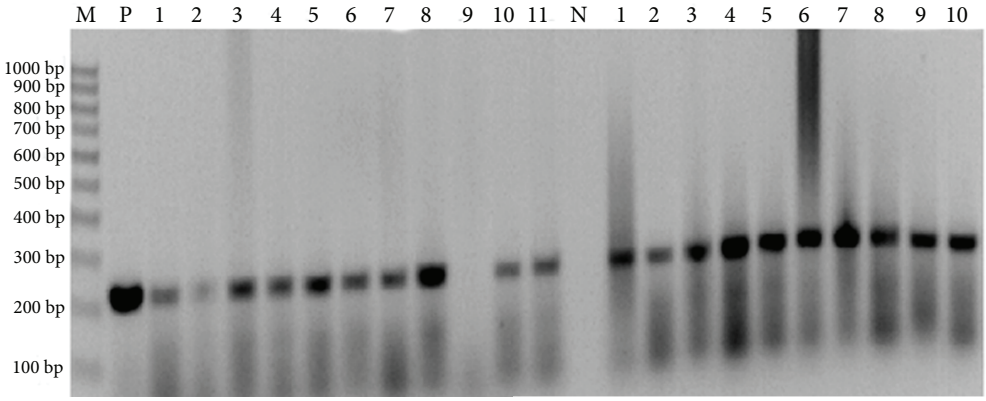

Figure 3. *bcsp*31-PCR amplification results of milk samples collected from Yukarı Danişment and Kırkgöze villages. M: marker, P: positive control, 1–11 Yukarı Danişment village milk samples; 1–10 Kırkgöze village milk samples.

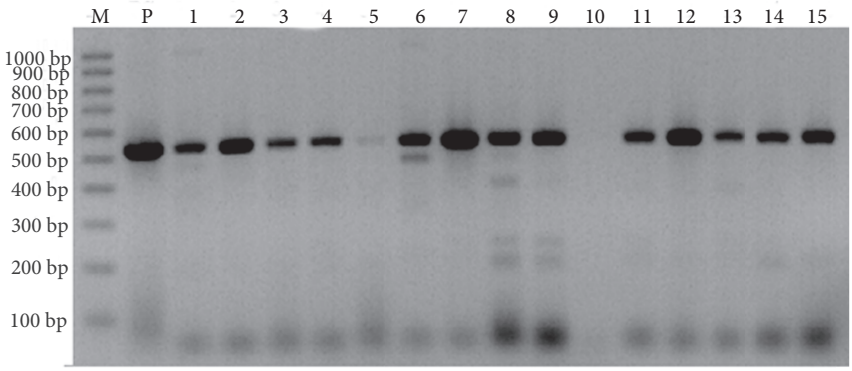

Figure 4. *omp*25-PCR amplification results of milk samples collected from Değirmenler village. M: marker, P: positive control, 1–15 Değirmenler village milk samples.

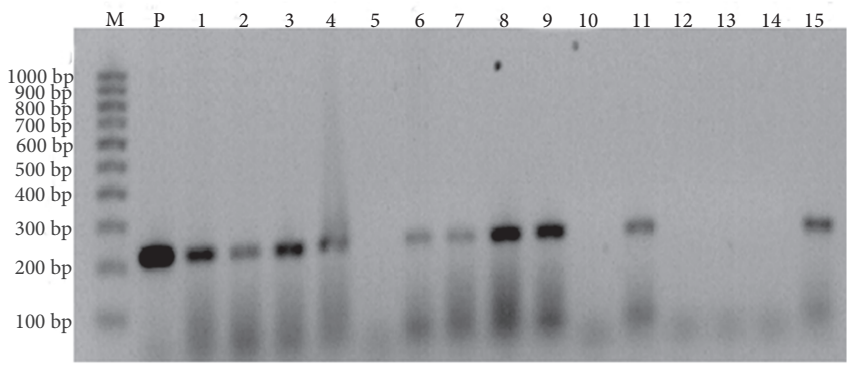

Figure 5. *bcsp*31-PCR amplification results of milk samples collected from Değirmenler village. M: marker, P: positive control, 1–15 Değirmenler village milk samples.

Table. The *bcsp*31-PCR (primers B4/B5) and the *omp*25-PCR (AF/AR primers) amplification results according to the distribution of settlements to milk samples.

Settlement	Number of samples	Number of positive samples	Primer pairs	Test results of positive samples			
				Number of positive samples and percentage		Number of negative samples and percentage	
Yeşilyayla village	20	19	B4/B5	16	84%	3	16%
			AF/AR	19	100%	0	0%
Yarımca village	20	18	B4/B5	16	89%	2	11%
			AF/AR	15	83%	3	17%
Küçüktüy village	20	17	B4/B5	16	94%	1	6%
			AF/AR	16	94%	1	6%
Çiftlik village	14	14	B4/B5	9	64%	5	36%
			AF/AR	11	79%	3	21%
Değirmenler village	15	13	B4/B5	10	77%	3	23%
			AF/AR	13	100%	0	0%
Dumlu village	11	10	B4/B5	9	90%	1	10%
			AF/AR	9	90%	1	10%
Tınazlı village	60	57	B4/B5	56	98%	1	2%
			AF/AR	51	89%	6	11%
Tebrizcik village	5	4	B4/B5	4	100%	0	0%
			AF/AR	4	100%	0	0%
Adnan Menderes village	1	1	B4/B5	1	100%	0	0%
			AF/AR	1	100%	0	0%
Ova village	1	1	B4/B5	1	100%	0	0%
			AF/AR	1	100%	0	0%
Büyükgeçit village	8	8	B4/B5	6	75%	2	25%
			AF/AR	8	100%	0	0%
Yukarı Danişment village	11	10	B4/B5	10	100%	0	0%
			AF/AR	9	90%	1	10%
Kırkgözeler village	10	10	B4/B5	10	100%	0	0%
			AF/AR	10	100%	0	0%
Ağcalar village	33	33	B4/B5	33	100%	0	0%
			AF/AR	30	91%	3	9%
Özbek village	3	3	B4/B5	3	100%	0	0%
			AF/AR	3	100%	0	0%
Alaca village	18	15	B4/B5	14	93%	1	7%
			AF/AR	13	87%	2	13%
Çukurca village	15	15	B4/B5	15	100%	0	0%
			AF/AR	14	93%	1	7%
Hınıs district	19	19	B4/B5	19	100%	0	0%
			AF/AR	19	100%	0	0%

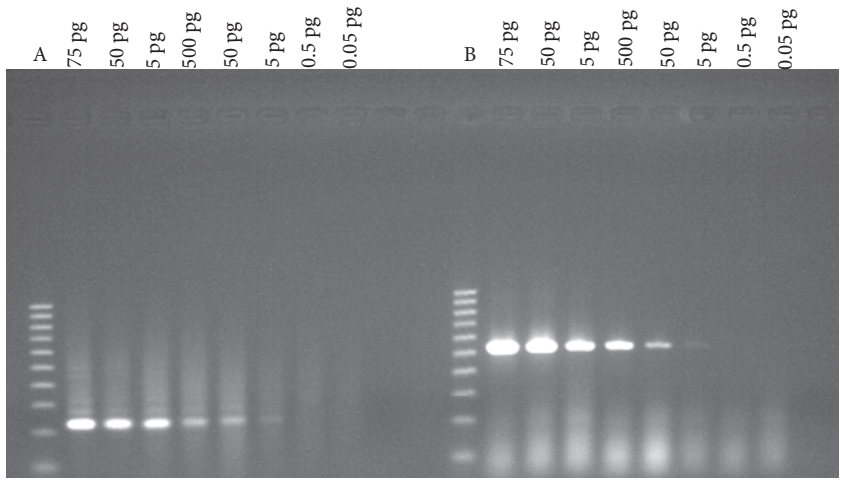

Figure 6. Susceptibility testing of total DNA isolation from milk. A: B4/B5 primer pair, 223-bp PCR product; B: AF/AR primer pair, 500-bp PCR product.

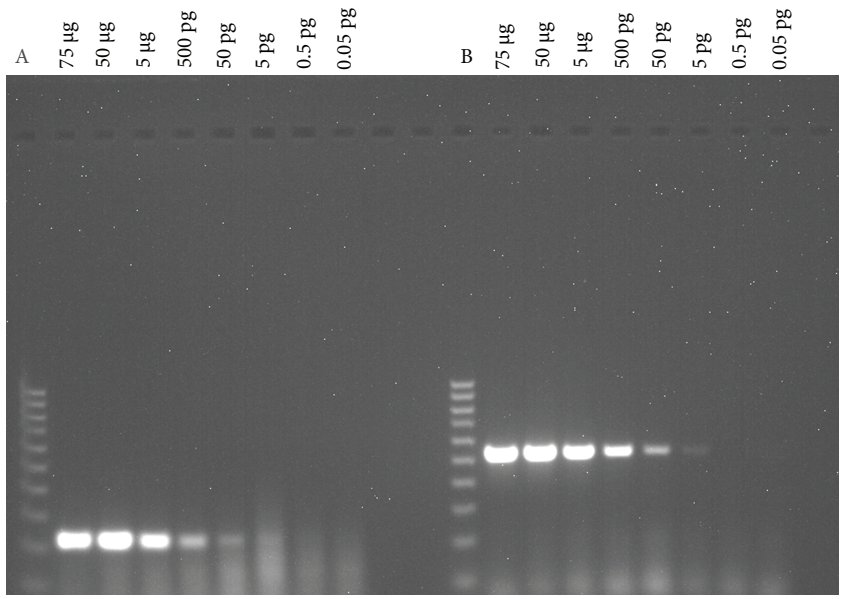

Figure 7. Susceptibility testing of DNA isolation from *B. abortus*. A: B4/B5 primer pair, 223-bp PCR product; B: AF/AR primer pair, 500-bp PCR product.

farm animals in our study, which is not surprising, since the animal husbandries and pasture areas in the region from which the samples were taken were highly contaminated with miscarriages, fecal waste, and the spread of animal byproducts and secretions.

This study found 17 of 334 milk samples to be negative. This may not mean that these animals are healthy or not infected with *Brucella* spp. The pathogen *Brucella* can be located in the lymph nodes of animals and may not be transferred or reach the milk during the time the sample is sample taken, or there may be a low number of bacteria present in these milk samples (19).

Our data have shown that both the DNA isolation procedure from milk and PCR amplification assays from *bcsp*31 and *omp*25 gene regions are accurate, reliable, and useful methods for the rapid diagnosis of brucellosis in cattle. When the risk of contamination during operation and the need for a short period of time for diagnosis of brucellosis in bovine milk are taken into consideration, the use of PCR is extremely advantageous. Experimental results can be reached within 4 h after acceptance of laboratory samples and extraction. This is a very short time period compared to conventional methods, and it also provides a significantly convenient, routine application. In addition,

it can be concluded that both the DNA extraction protocol and PCR assay used in our study would be appropriate and useful for brucellosis epidemiological research in Turkey.

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