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RESEARCH ARTICLE

Evaluation of the Analytical Efficiency of Real-Time PCR in the Diagnosis of Brucellosis in Cattle and Sheep

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Abstract

Brucellosis is an important infectious disease that affects animal and public health in many developing countries, including Turkey. The control and eradication of brucellosis are contingent upon methods that provide a fast and reliable diagnosis. In this context, molecular methods which enable the enzymatic amplification of bacterial conserved gene regions are advantageous. In this study, samples of cattle and sheep blood serum sent to our laboratory from enterprises at risk of brucellosis in different settlements of the Central Anatolia and the Black Sea Regions of Turkey were analyzed. In our study, we aimed to investigate brucellosis using serological methods and Real-Time PCR (RT-PCR), and to analyze these methods comparatively. To this end, RBPT, SAT and CFT tests, as well as the Brucella RT-PCR, which enables the amplification of the BCSP31 gene found in all Brucella species, were used. In the 368 serum samples analyzed, Brucella positivity was determined as 11.41%, 10.05% and 9.8% by RBPT, SAT and CFT, respectively, while the RT-PCR gave the same rate of positivity (9.8%) as CFT. When CFT is taken as a reference test, the sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of the RT-PCR were determined as 100%; and it was found to be perfectly compatible with CFT, with a kappa value of 1.000. The number of bacterial genomes that could be detected by the RT-PCR in the presence of DNA of the Brucella melitensis biotype 3 (Ether) reference strain was determined as 3.94x103 copies. Linear regression analysis revealed that the amplification efficiency (92.71%) of the RT-PCR was within the desired limits (90-110%) and that the RT-PCR was repeatable (CV 2.9%) and reproducible (CV 1.8%). This study indicates that the RT-PCR is a useful method for application in the diagnosis of bovine and sheep brucellosis thanks to its high analytical efficiency; it also emphasizes the importance of blood serum samples as preferable clinical materials in this context.

Keywords: *Brucella spp., Brucellosis, Cattle, Sheep, Real-Time PCR, Analytical competence*

Sığır ve Koyunlarda Brusellozisin Tanısında Real-Time PCR'nin Analitik Yeterliliğinin Değerlendirilmesi

Öz

Brusellozis, ülkemizle birlikte gelişmekte olan birçok ülkede hayvanlarda ve halk sağlığı açısından önem arzeden infeksiyöz bir hastalıktır. Brusellozisin, kontrol ve eradikasyonunda hızlı ve güvenilir teşhis imkânı sunan yöntemlere her zaman ihtiyaç duyulmaktadır. Bu kapsamda, bakterilerin korunaklı gen bölgelerinin enzimatik amplifikasyonunu sağlayan moleküler yöntemler avantaj sağlamaktadır. Bu çalışmada, İç Anadolu ve Karadeniz Bölgelerine ait farklı yerleşim birimlerindeki brusellozis riski taşıyan işletmelerden laboratuvarımıza gönderilen sığır ve koyunlara ait kan serum örnekleri incelenmiştir. Çalışmamızda, brusellozisin serolojik yöntemler ve Real-Time PCR (RT-PCR) ile araştırılması ve bu yöntemlerin karşılaştırmalı analizi amaçlanmıştır. Bu amaçla, RBPT, SAT ve KFT testleri ile birlikte tüm Brucella türlerinde bulunan BCSP31 geninin amplifikasyonunu sağlayan Brusella RT-PCR metodu kullanılmıştır. İncelenen 368 serum örneğinde RBPT, SAT ve KFT ile Brucella pozitifliği sırayla %11.41, %10.05 ve %9.8 belirlenirken, RT-PCR analizi sonucu, KFT ile aynı oranda (%9.8) pozitiflik elde edilmiştir. KFT referans test olarak dikkate alındığında, RT-PCR'nin sensitivite, spesifite, pozitif ve negatif prediktif değerleri ve tanı doğruluğu %100 ve 1.000'lık kappa değeri ile KFT ile mükemmel derecede uyumlu olduğu saptanmıştır. Brucella melitensis biyotip 3 (Ether) referans suşuna ait DNA eşliğinde RT-PCR ile saptanabilen bakteriyel genom sayısı 3.94x103 kopya olarak belirlenmiştir. Gerçekleştirilen linear regresyon analizi ile RT-PCR'nin amplifikasyon verimliliği, istenen sınırlar (%90-110) arasında (%92.71) yer almış ve tekrarlanabilir (CV %2.9) ve üretilebilir (CV %1.8) olduğu belirlenmiştir. Bu çalışma ile yüksek analitik verimliliği sayesinde RT-PCR'nin sığır ve koyun brusellozisin teşhisinde başvurulabilir bir yöntem olduğu öngörülmüş ve bu kapsamda kan serum örneklerinin tercih edilebilir klinik materyaller olarak önemi vurgulanmıştır.

Anahtar sözcükler: *Brucella spp., Brusellozis, Sığır, Koyun, Real-Time PCR, Analitik yeterlilik*

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Introduction

Brucellosis, caused by bacteria of the *Brucella* genus, is a disease that affects many animal species such as ruminants, pigs, dogs, foxes, and some marine mammals. Capable of being transmitted directly or indirectly to humans, it poses a risk to public health and is known as the most common zoonotic disease in the world. The predominant *Brucella* species in cattle is *Brucella abortus*, and in sheep *Brucella melitensis* [1]. These agents mostly settle in the uterus, placenta, fetus, and breast tissue in animals, and cause necrotic and inflammatory infections which lead to abortion, infertility and mastitis in affected animals. In addition to causing significant economic losses in the livestock industry through reduced fertility, decreased animal product and quality, and expensive protection and control practices [2].

Definitive diagnosis is important for the control of *Brucella* infection in animals and humans. Clinical diagnosis is generally based on the presence of reproductive system infections in livestock. However, this hypothetical diagnosis must be confirmed by laboratory methods. In this context, culture methods have been reported as the "gold standard" in the diagnosis of brucellosis. However, since the culture method is time-consuming and the *Brucella* agents have to be studied in biosafety level 3 laboratories, culture procedures are not always available. Serological tests are frequently used in the implementation of control and eradication programs of brucellosis. Some of them (Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), etc.) are used as screening tests, while others (2-Mercaptoethanol, Complement Fixation Test (CFT) and ELISA) are used as confirmation tests. With its high diagnostic accuracy, CFT is used to confirm the diagnosis of *B. abortus* and *B. melitensis* infections and is recommended by the World Animal Health Organization (OIE) as a reference test for international animal mobility [3]. Although they have been continuously improved in terms of sensitivity and specificity, these tests have some disadvantages, such as their high cost, the need for special equipment and expert personnel, and the inability to distinguish between vaccinated and naturally infected animals. Molecular techniques are advantageous tools for the direct diagnosis of brucellosis and for the verification of agents identified by conventional methods, and can also be used in the investigation of bacterial variants and vaccine candidates, and to perform virulence assessment and epidemiological analysis [4]. Recently, several PCR methods have been reported that enable the amplification of conserved gene regions of *Brucella* agents, such as the 31-kDa surface protein *(BCSP31), 16S rRNA*, and the insertion sequence *IS711* [4-6]. Among these, Real-Time PCR (RT-PCR) method, which provides results simultaneous with the enzymatic amplification cycle of the target gene region, offers a more advantageous diagnosis with features such as high sensitivity and amplification efficiency, a short turnaround time, and no need for electrophoresis imaging [6,7].

This study aimed to evaluate the analytical efficiency of an *in-house* RT-PCR for the rapid and sensitive diagnosis of the *Brucella* genome in bovine and sheep blood sera. In this context, a comparative analysis was carried out between the test and the RBPT, SAT and CFT methods.

Material and Methods

Ethical Permission

The ethical permission of the study was ensured by the decision of The Republic of Turkey Ministry of Agriculture and Forestry, Veterinary Control Central Research Institute, Animal Experiments Local Ethics Committee with the code of "2021-01".

Study Material and Sampling

This study was carried out on blood serum samples from 368 animals, 311 cattle and 57 sheep, sent to the laboratories of the Veterinary Control Central Research Institute from enterprises with a recent history of abortion in the Central Anatolia and Black Sea Regions of Turkey *(Table 1)*. There was no history of vaccination against *Brucella* agents in the cattle and sheep whose blood samples were taken in the study. Abortion cases usually correspond to the last few

Table 1. *DNA concentrations and predicted genome numbers of positive control subdilutions*

Dilution	dsDNA Concentration (ng/µL)	The Number of Copies in 1 µL	The Number of Copies in 2 µL	Average Ct (±SD)	Coefficient of Variation (CV, %)	
					Intra-assay	Inter-assay
Stock	19.2	$5.4x10^6$	$1.08x10^7$	20.79±0.14	0.4	0.3
10^{-1}	1.3	$3.66x10^5$	$7.32x10^5$	21.88±1.21	6.4	6.4
10^{-2}	0.9	$2.53x10^5$	$5.06x10^5$	27.44±0.56	2.7	0.7
10^{-3}	0.1	$2.81x10^4$	$5.62x10^4$	31.26±0.26	0.8	1.2
10^{-4}	0.01	$2.81x10^03$	$5.62x10^3$	33.82±0.35	1.2	1.1
10^{-5}	0.007	$1.97x10^3$	$3.94x10^3$	35.83±0.93	3.6	1.1
10^{-6}	0.0004	$1.12x10^2$	$2.24x10^2$	ND*	-	-

** **Not determined:** Ct value was taken as ≥36 in regression analysis for samples that do not form an amplification curve and are considered negative*

months of pregnancy in animals, and blood samples were taken at 2 to 4 weeks following the abortion. The blood samples were centrifuged at 3.000 rpm for 10 min; one aliquot of the obtained serum samples was stored at -80°C for serological analysis and another for the RT-PCR.

RT-PCR Analysis

DNA extraction from samples: DNA extraction from serum samples was carried out using a commercially available nucleic acid purification kit (QIAampDNA Mini Kit, Qiagen, Germany) in accordance with the manufacturer's instructions. DNA concentrations were determined by measuring at 260 and 280 nm wavelengths with a NanoDrop (ND-1000 spectrophotometer, Marshall Scientific, USA).

Analysis of samples: The RT-PCR analysis of the serum samples was performed on a RT-PCR Detection System (CFX96, BioRad, USA). A RT-PCR test (Bioeksen Ar-Ge Teknolojileri Ltd.® Istanbul) that enables the amplification of the *BCSP31* gene region of *Brucella* species was used. The reaction mixture prepared in a volume of 11 µL for each sample was composed of 5 µL RT-PCR master mix, 3 µL *Brucella* spp. oligo mix, 1 µL internal control DNA and 2 µL template DNA components. The thermal cycle was set as 5 min pre-denaturation at 95°C, 45 cycles consisting of denaturation at 95°C for 15 sec, and binding and elongation at 60°C for 40 sec. In the RT-PCR, FAM-labeled *Brucella* spp. targeted oligonucleotides and HEX-labeled internal control (IC), which provides sample-based inhibition control and kit reagent control, were used. Thus, two amplification curves were obtained for each positive sample and only IC results for negative samples. All positive and negative serum samples were studied in duplicate. The RT-PCR was performed in the presence of negative control (RNAse/DNAse free water) and positive control (in the kit). Samples with no amplification or a cycling threshold (Ct) of 36 or greater were considered negative.

Analytical capability of the RT-PCR: The *Brucella melitensis* biotype 3 (Ether) reference strain (NCTC 10505) was used to test the analytical ability of the RT-PCR. Bacterial DNA extraction was performed with a commercially available nucleic acid purification kit (QIAampDNA Mini Kit, Qiagen, Germany). The total amount of double-stranded DNA (dsDNA) was determined using a NanoDrop ND-1000 spectrophotometer and the total number of DNA copies was then calculated using an interactive program [8] with the formula: DNA copy number=(DNA amount (ng) x $6.022x10^{23}$)/(DNA length (bp) x $1x10^{9}$ x 650 Dalton). In this calculation, the DNA length of *B. melitensis* was taken as 3.294.931 bp [9]. Intra-assay repeatability was tested by calculating Ct values following three amplifications of stock and 10^{-5} DNA copies of the *B. melitensis* biotype 3 (Ether) by the RT-PCR. Inter-assay reproducibility was determined by the Ct values obtained as a result of the amplification of positive control DNA and its sub-dilutions on two different days by the RT-PCR.

Serological Analysis

Rose Bengal Plate Test (RBPT): The RBPT was conducted according to the method reported by Alton et al.[10]. The test was carried out using the Brucella RBPT antigen produced at the İstanbul Pendik Veterinary Control Institute of the Ministry of Agriculture and Forestry of Turkey. The antigen was used to detect IgG specific for Smooth *Brucella* species *(B. abortus, B. melitensis* and *B. suis)*. 15 µL of the RBPT test antigen was dropped on a clean slide. 15 µL of the serum sample to be tested was taken and dropped next to the antigen. After mixing the antigen and serum samples, the reaction that occurred within 4-5 min on the slide was evaluated with the naked eye. Large/small precipitate (agglutination) like sand grains formed on the slide was evaluated as positive. The absence of any agglutination on the slide with the serum-antigen mixture remaining as a homogeneous suspension was evaluated as negative.

Serum Agglutination Test (SAT): The SAT was performed according to the method reported by Alton et al.[10]. In the test, Brucella Tube Agglutination Test Antigen, produced at the İstanbul Pendik Veterinary Control Institute of the Ministry of Agriculture and Forestry of Turkey, was used. The antigen was used to detect IgM and IgG in blood serum for smooth *Brucella* species *(B. abortus, B. melitensis* and *B. suis)*. The SAT was performed in the presence of positive and negative control sera. The lace-like precipitate (agglutination) formed at the bottom of the glass tubes was evaluated as positive. Dilution in the last tube in which the precipitate was seen was considered the serum antibody titer. During the evaluation, the degree of agglutination was expressed in IU and a serum sample containing 30 or more IU was considered positive.

Complement Fixation Test (CFT): The CFT was conducted according to the method reported by Alton et al.[10]. After preparing a 5-fold sub-dilution of the serum samples to be tested in strip tubes with Veronal buffer (VB), the samples were inactivated for 50 min in a 58°C water bath. 25 µL of VB was added to the wells on the microplate and 25 µL of serum samples were added to the wells. 25 µL of test antigen (CFT antigen, Istanbul Pendik Veterinary Control Institute of the Ministry of Agriculture and Forestry of Turkey) was added to wells from A to G. 50 µL of complement (Freeze-dried Guinea Pig Complement ID. Vet, France) was added to the wells from A to H. After incubation, 50 µL hemolytic system consisting of sheep erythrocytes (2%) and amboceptor (Virion/Serion 1: 1500 AMB) was added to the wells. The test limit titer was determined by observing the lysis occurring in the microplate wells. Serum dilutions containing 20 or more International Complement Fixation Units (ICFTU) per milliliter were accepted as positive [11].

Statistical Analysis

Programs enabling interactive calculation were used in the statistical analysis of the data. The sensitivity, specificity and

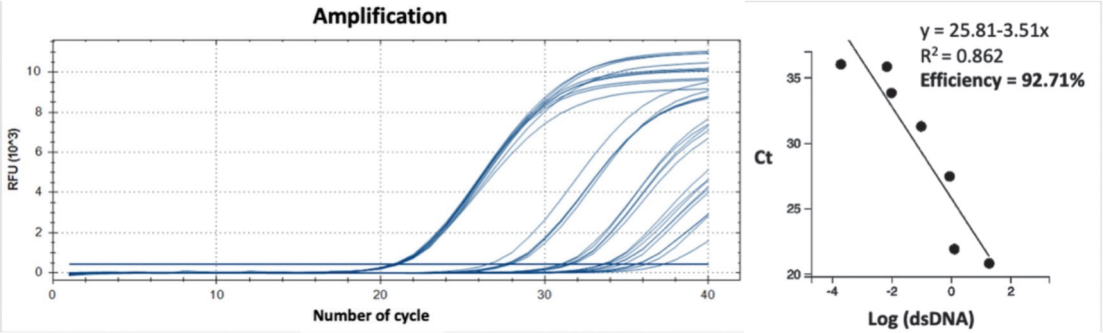

Fig 1. Amplification curves and linear regression analysis of the DNA dilutions of *B. melitensis* biotype 3 (Ether) strain

Table 2. *Distribution of samples according to settlements*

Province	Number of Samples	RT-PCR Positivity		P- Value
		n	%	
Çankırı	48	6	12.5%	>0.05
Ankara	189	19	10.05%	
Çorum	31	3	9.68%	
Kırşehir	12	0	0%	
Yozgat	37	3	8.11%	
Kastamonu	21	3	14.29%	
Karabük	5	1	20%	
Bartın	9	0	0%	
Nevşehir	7	0	0%	
Kırıkkale	9	1	11.11%	
Total	368	36	9.78%	

diagnostic accuracy analyzes of the tests were performed with the reported program [12]. Linear regression analysis was carried out with the qPCR Library Quantification program [13]. The coefficients of variation for the test repetitions (CV) were calculated with the reported program [14]. The Chi-square test was carried out with the reported program [15].

Results

RT-PCR Findings and Analytical Capability

The results of the RT-PCR revealed that 36 (9.78%) of 368 serum samples contained *Brucella* spp. DNA *(Fig. 1)*. The amount of template DNA (2 µL) used in the RT-PCR, the gene copy number of the *B. melitensis* biotype 3 (Ether) DNA dilutions between the stock and 10^{-6} varied $1.08x10^7$ to $2.24x10^2$. Thus, the number of bacterial genomes detectable (in other words limit of detection (LOD)) by the RT-PCR was calculated as $3.94x10^3$ copies, with a standard deviation of 0.93 and a variation coefficient of 0.026. In the linear regression analysis performed, the amplification efficiency (92.71%) of the RT-PCR was determined to be within the desired amplification efficiency limits (90-110%) with a y-cutpoint of 25.81 and a determination coefficient (R^2) of 0.862 *(Fig. 1)*. The intra-assay repeatability of the RT-PCR was determined by calculating the average coefficient of variation (CV), obtained by amplifying the *B. melitensis* biotype 3 (Ether) strain three times using different DNA dilutions between the stock and 10^{-5}; the average CV was determined as 2.9%. The inter-assay reproducibility of the RT-PCR was determined by calculating the average coefficient of variation (CV), obtained by amplifying the *B. melitensis* biotype 3 (Ether) strain two times on two different days with the stock to 10^{-5} sub-dilutions of control DNA; the average CV was determined as 1.8% *(Table 1)*.

Serological Analysis Findings

In this study, 42 (11.41%) of 368 blood serum samples were found to be positive in terms of *Brucella* spp. by RBPT, 37 (10.05%) by SAT and 36 (9.78%) by CFT *(Table 2)*. It was found that there was no significant difference between the serological tests in determining *Brucella* antibodies in cattle and sheep blood serum samples (Chi-square=0.602 and P=0.740). All samples that were found to be positive with CFT were also found positive by the RT-PCR. When CFT was taken as the reference test, the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of the RT-PCR were determined as

Table 3. *Diagnostic values of tests for cattle and sheep sera*

Test		Serological Tests		Analytic Diagnostic Values												
		Positive	Negative	Sensitivity		Specifity		PPD		NPD		Diagnostic Accuracy		Kappa		
				%	%95 CI	%	%95 CI	%	%95 CI	%	%95 CI	%	%95 CI	%	%95 CI	SE
RT-PCR	**RBPT**															
	Positive	36	0	85.71	71.46-94.57	100	98.87-100	100		98.19	96.28-99.13	98.37	96.49-99.40	0.914	0.846-0.982	0.035
	Negative	6	326													
	SAT															
	Positive	36	0	97.30	85.84-99.93	100	98.89-100	100		99.70	97.95-99.96	99.73	98.50-99.99	0.985	0.955-1	0.015
	Negative	1	331													
	CFT															
	Positive	36	0	100	90.26-100	100	98.90-100	100		100		100	99-100	1.000		
	Negative	0	332													

PPD: *positive predictive value,* ***NPD:*** *negative predictive value,* ***CI:*** *confidence interval,* ***SE:*** *standard error*

100%. It was observed that a perfect match was achieved, with a kappa value of 1.000, between the RT-PCR and CFT, which is used as a reference test in the detection of *Brucella* infections *(Table 3)*.

Discussion

Brucellosis is a zoonotic disease that is endemic in many parts of the world, including Turkey, and adversely affects animal production and public health. Brucellosis, which is rarely fatal, is highly contagious. In this respect, there is always a need for fast and reliable diagnostic methods to be used in the follow-up and eradication of the disease. Although the culture method that allows isolation of the agent is still known as the "gold standard" in the diagnosis of brucellosis, it is not widely used due to the time-consuming and biohazardous characteristics. The practical diagnosis of brucellosis in animals is mainly based on antibody-based serological methods. However, these methods have many negative features such as cross-reactions, which caused by vaccinated animals or close bacteria harbouring the same antigenic structures, restrict their use in routine diagnosis as mentioned [1,7]. The 31-kDa surface protein *(BCSP31)* gene is the most protected gene region among the *Brucella* members and has found widespread use in molecular diagnosis of infection in humans and animals [6,16]. In this study, an *in-house* RT-PCR kit was found to be lower ($3.94x10^3$ copies) than in the other studies [7,16,17]. This can be interpreted with reference to the use of low cut-off values of the RT-PCR that allows the detection of DNA with a lower copy number as well as the kit differences. The average coefficients of variation (CV) of the intra-assay repeatability and the inter-assay reproducibility of the RT-PCR were determined as 2.9% and 1.8%, respectively, and these values are below the acceptable level (<10%) for the RT-PCR [18]. This indicates that the test repetitions are consistent, hence the method is reproducible. In an optimized RT-PCR, it is desirable that the coefficient of detection is greater than 0.980, the amplification efficiency ranges between 90% and 110%, and the reaction repeats are consistent [19]. In this study, a value (0.862) close to the ideal determination coefficient (R^2) was observed and the amplification efficiency (92.71%) of the RT-PCR was found to lie within the acceptable limits [19]. Moreover, the amplification efficiency of the test is very similar to those high efficacy was reported for the BCSP31 gene region of *B. melitensis* bacteria [20-22].

Many studies have been reported on the diagnostic capability of the RT-PCR, which enables amplification of the *BCSP31* gene. In these studies, performed in humans and animals, analytical calculations such as diagnostic sensitivity and specificity were performed and compared with cultural and/or serological methods [21,23]. In this study, the diagnostic efficiency, sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy of the RT-PCR were determined as 100%, when compared with the sample-focused approach and CFT. A perfect agreement (kappa was 1.000) was found between CFT and RT-PCR. Although there are some small proportional differences in diagnostic competence, it is generally similar to the other studies [20,21]. When compared to the RBPT and SAT, the diagnostic competence values of the RT-PCR PCR are slightly lower, except for the specificity and positive predictive value. However, the agreement between the RT-PCR and these tests is still close to the perfect agreement (kappa was 0.914 and 0.985, respectively).

Brucella spp. are facultative intracellular microorganisms and they settle in macrophages. After completing replications in these cells, they migrate to the lymphoid tissues of the reproductive system as a result of a primary bacteremia. The agent causes a secondary bacteremia from these tissues, which subsequently leads to a generalized infection and then abortion. Therefore, these persistent bacteremia phases, which almost always contain the microorganisms in the bloodstream, are repeated in the next gestational period [24]. Modern PCR methods are

capable of detecting both living bacteria and bacteria that have been phagocyted or killed by macrophages in different compartments of the blood during the periods of bacteremia [25,26]. Also, PCR techniques have the ability to determine the course of the infection. In this context, blood in which bacteria-laden macrophages are constantly circulating is a useful clinical material that can be used for diagnosis as a source of DNA belonging to the infectious agent [25]. Different diagnostic advantages of different blood compartments such as whole blood or serum have been reported. Although the use of a whole blood compartment containing leukocytes is essential for the obligate intracellular agents, there is no such requirement for the facultative intracellular bacteria such as *Brucella* species. In addition, the analytical sensitivity of the serum in the diagnosis of brucellosis in humans and animals is comparable with the whole blood phase. Blood serum is the preferred DNA source in PCR because it provides quick test results and simplifies the method, and does not contain inhibitory structures such as anticoagulants and hemoglobin [25,26]. In this respect, the use of samples of blood serum from animals at risk of brucellosis in this study has once again confirmed it as an evaluable clinical material in the diagnosis of brucellosis. Based on the above-mentioned high diagnostic characteristics of the RT-PCR and the usability of the blood serum samples as a diagnostic clinical material, *Brucella* positivity in cattle and sheep was found to be 9.78% for the region studied. Due to the heterogeneous sample distribution, there was no statistical relationship between the disease prevalence and the provinces sampled (P>0.05). Nevertheless, the positivity for *Brucella* obtained on the provincial basis is similar to that [27] reported by the Veterinary Control Central Research Institute between 2007 and 2011, and values close to 2011 seropositivity were obtained in particular.

The control and eradication of brucellosis in animals in Turkey is based on the vaccination of susceptible animals and the slaughtering of infected animals [11]. In this respect, it is important to use reliable and validated test methods that provide rapid laboratory diagnosis. In this study, the analytical competence of an *in-house* RT-PCR was tested and it was determined that this method with its high diagnostic efficiency can be of benefit in the diagnosis of brucellosis in cattle and sheep populations. In addition, the importance of blood serum as a preferable clinical material in the molecular diagnosis of brucellosis in animals was reinforced.

Conflict of Interest

The authors did not report any conflict of interest related to this article.

Author Contributions

DKY and DA planned, designed, and supervised the research procedure, carried out the experiments and the analytic tests and wrote the article.

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