Screening *Brucella* spp. in bovine raw milk by real-time quantitative PCR and conventional methods in a pilot region of vaccination, Edirne, Turkey

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ABSTRACT

Brucellosis is a worldwide zoonotic disease transmitted to humans by consumption of contaminated milk and milk products. Brucellosis is endemic in Turkey, and Edirne has a high *Brucella* prevalence. Brucellosis is prevented by live-attenuated vaccines for animals and the vaccination program has been in place since 1984 in Turkey. Thrace is the pilot region for this vaccination program. The gold standard diagnostic technique for brucellosis is still the isolation of suspicious bacterial colonies followed by bacteriological identification, but it is very time consuming and laborious. In many studies, *Brucella* has been investigated by PCR techniques. However, PCR-based methods cannot differentiate between the vaccine strain and the virulent strain; thus, the vaccine strain may interfere with the virulent strain and causes false-positive reactions. To monitor brucellosis control programs effectively, it is important to distinguish vaccine and field strains of *Brucella* spp. In this study, raw milk samples were collected from 99 cows at 12 different barns in 5 villages of Edirne (Turkey). Bacteriological analyses and real-time quantitative (q)PCR experiments were applied to all samples. The DNA was isolated using Biospeedy DNA-Tricky Purification Kit (Bioeksan, Istanbul, Turkey). For all reactions, Roche Light Cycler Nano (Roche Diagnostics, Mannheim, Germany) instrument and Biospeedy EvaGreen qPCR Pre-Mix (Bioeksan) were used. The data were analyzed using Roche LightCycler NanoSoftware 1.0. For samples that were negative by bacteriological analyses and positive by qPCR, we developed a novel qPCR-based method to differentiate the virulent *B. abortus* strains and *B. abortus* S19 vaccine strain. We designed qPCR primers targeting the outer membrane protein of *B. abortus*. The qPCR products were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on

an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). In total, 2.02% of the samples were *Brucella* positive, by both bacteriological method and the novel qPCR method. We concluded that, to obtain true-positive results in *Brucella* spp. screening studies for milk, differentiating the virulent and vaccine strain should not be disregarded.

Key words: *Brucella* spp., milk, qPCR, virulent strain, vaccine strain

INTRODUCTION

Brucellosis is a worldwide zoonotic disease (Chacón-Díaz et al., 2011; Najum et al., 2012; Pacheco et al., 2012; Arasoglu et al., 2013; Qasem et al., 2015) transmitted to humans by consumption of contaminated milk and milk products (Kaynak-Onurdag et al., 2008). Brucellosis causes economic losses including reproductive failure and abortion in animals (Vemulapalli et al., 1999; Smirnova et al., 2013). *Brucella* spp. may also cause highly diverse and severe illnesses in humans that are very difficult to diagnose and treat (Moussa et al., 2011). Among the genus *Brucella*, *Brucella abortus* is the most common strain infecting cattle (Amin et al., 2012).

In Turkey, brucellosis is endemic and *Brucella* prevalence is high in Edirne city (Aytekin et al., 2011; Calik and Gokdeniz, 2011). Brucellosis can be prevented by live-attenuated vaccines for animals (Kaynak-Onurdag et al., 2008; Nan et al., 2014). In Turkey, the vaccination program has been held since 1984 (Abdelkareem et al., 2011). Thrace is the pilot region for this vaccination program; however, brucellosis continues to be endemoepidemic in this region (Aytekin et al., 2011). Many dairy companies supply their milk from the cows in this region; thus, screening milk for *Brucella* contamination becomes very important. The S19 vaccine was developed for the immunization of cattle, and the traditionally recommended immunization procedure by World Health Organization (1986) has been administration of the vaccine via the subcutaneous route in female calves at 4 to 8 mo of age. However, conjunctival

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administration of the vaccine to a herd or flock is also a practical and effective procedure (WHO, 2006). In Turkey, the route of administration has been changed according to the "Control and eradication of *Brucella* with conjunctival vaccine" project of the Republic of Turkey Ministry of Food, Agriculture and Livestock, and all young and adult cattle have been vaccinated by conjunctival live-attenuated *B. abortus* S19 vaccine since 2012 (Republic of Turkey Ministry of Food, Agriculture and Livestock, 2012).

The gold standard diagnostic technique of brucellosis is still the isolation of suspicious bacterial colonies from host tissues, milk, or vaginal exudates, followed by bacteriological identification (Alton et al., 1988; Tiwari et al., 2014). However, this method is very time consuming and laborious (Tiwari et al., 2014). Polymerase chain reaction is a fast and reliable diagnostic method with the advantage that it can lead to earlier detection and it can detect low numbers of cells or targeted gene copies (Kumar et al., 2008; Amin et al., 2012; Arasoglu et al., 2013). Therefore, in many studies, *Brucella* has been investigated by PCR techniques (Hamdy and Amin, 2002; Xiao et al., 2005; O'Leary et al., 2006; Alamian et al., 2014; Wareth et al., 2014). However, PCR-based methods cannot differentiate the vaccine and the virulent strain; thus, the vaccine strain may interfere with virulent strain and result in false-positive reactions. To monitor brucellosis control programs effectively, it is important to distinguish the vaccine and field strains of *Brucella* spp. (Fallah et al., 2009; Nan et al., 2014). *Brucella abortus* S19 vaccine strain may be found in cow milk according to the reproductive period of cows, causing positive PCR results and bacteriologically negative results (Fallah et al., 2009). For this reason, for *Brucella* spp. screening studies in milk, differentiating the virulent and vaccine strain is important.

In this study, our aim was to investigate *Brucella* spp. in milk obtained from cows in Edirne in the Thrace region of Turkey and compare bacteriological and molecular methods for screening *Brucella* and differentiating the virulent and vaccine strains.

MATERIALS AND METHODS

Raw milk samples were collected from 99 cows at 12 different barns in 5 different villages of Edirne (Turkey), which is situated near the border of Bulgaria and Greece (41°40'41"N, 26°33'49"E) in the Thrace region.

Cows were milked by hand before they were transferred to the milking machine. Before milking, the teats were cleaned with clean warm water and the first milk was disregarded from every teat. With a mixture

of milk from each teat of an udder, 250 mL of milk (pooled) was obtained from each cow.

Bacteriological Analyses

Farrell broth (FB) was prepared as the pre-enrichment medium using *Brucella* Broth Base (BBB; BD Diagnostic Systems, Franklin Lakes, NJ), *Brucella* Selective Supplement (BSS; SR0083, Oxoid, Wesel, Germany), glucose (Merck, Darmstadt, Germany), and inactivated horse serum. To prepare inactivated horse serum, horse blood was obtained from Istanbul University Faculty of Veterinary Medicine. It was incubated at room temperature for 3 h and centrifuged at $2,800 \times g$ for 10 min. Serum was separated and inactivated at 56°C for 30 min and kept at -20°C until use. Before adding to the medium, inactivated horse serum was thawed at 37°C in a water bath and filtered through a 0.4-μm-pore-sized filter. The BBB was prepared according to the instructions, and 1.5% glucose was added to the medium before sterilization. After autoclave sterilization at 121°C for 15 min, the medium was cooled to 45 to 50°C and 5% inactivated horse serum and 10 mL of BSS were added per 1 L of medium. The medium was apportioned to sterile test tubes in 9-mL aliquots and kept at 4°C until use. Farrell agar (FA) was prepared with the same procedure of FB, using *Brucella* Agar Base (BD Diagnostic Systems) instead of BBB as the base medium (Farrell, 1974; Taşçı and Kaymaz, 2009).

As soon as the milk samples reached the laboratory, 1 mL of each sample was transferred to the 9-mL FB tubes. Double transfer was applied for every milk sample for incubation both in 10% CO₂ conditions and aerobic conditions. Cultures were incubated at 37°C for 5 to 7 d. A *Brucella melitensis* isolate obtained from Trakya University Health Center for Medical Research and Practice (Hospital) was incubated under the same conditions as a positive control of the medium. After incubation, a loopful of culture was transferred to 2 FA plates, one for incubation in 10% CO₂ and the other for incubation in aerobic conditions. Plates were incubated at 37°C for 5 to 7 d. Suspected *Brucella* colonies were subjected to oxidase, catalase, and urease tests following Gram staining. The oxidase test was performed with 6% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma, Munich, Germany) prepared with dimethyl sulfoxide (Merck). For the catalase test, 3% H₂O₂ (Riedel de Haën, Germany) solution was used. Urea agar base (Merck) including phenol red (Merck) was sterilized, and urea (Sigma, Germany) was added to the medium after being cooled to 45 to 50°C. Slant agar tubes were prepared and used for testing urease activity. Lead acetate test strips (Merck) were used for

Table 1. Primers, targets, and references

Primer	Sequence (5'–3')	Target	Reference
Babo_VF	GGGAATAGCAAGGAGCAAGG	Outer membrane protein of <i>Brucella abortus</i>	This study
Babo_VR	CAACAGAACCATGAGCGGAA	(GenBank ID: BABS19 I00660)	This study
Bact_F	AGAGTTTGATC(CA)TGGCTCAG	All bacteria	Lane (1991)
Bact_R	AAGGAGGTGATCCAGCCGCA	All bacteria	Lane (1991)
Bmel_F	TGCGATCGGCAGTTTCAA	<i>Brucella melitensis</i>	Hinić et al. (2008)
Bmel_R	CCAGCTTTTGGCCTTTTCC	<i>B. melitensis</i>	Hinić et al. (2008)
Babo_F	GCACACTCACCTTCCACAACAA	<i>B. abortus</i>	Hinić et al. (2008)
Babo_R	CCCCGTTCTGCACCAGACT	<i>B. abortus</i>	Hinić et al. (2008)
Bovi_F	GCCTACGCTGAAACTTGCTTTTG	<i>Brucella ovis</i>	Xavier et al. (2010)
Bovi_R	ATCCCCCATCACCATAACCGAAG	<i>B. ovis</i>	Xavier et al. (2010)

the H₂S test. Bacteria were inoculated to BBB and a lead acetate paper strip was inserted between the plug and inner wall of tube, above the inoculated medium, and incubated at 35°C. When the strip turned black, the strip was changed and the duration of H₂S production was determined.

DNA Isolation

The DNA was isolated using Biospeedy DNA-Tricky Purification Kit (Bioeksan, Istanbul, Turkey). Frozen milk samples were thawed at room temperature. Five hundred microliters of a milk sample (triplicates for each sample) was transferred to a 1.5-mL tube containing 300-mg metal beads with a radius of 1 mm. Samples were homogenized for 2 min at 7,000 rpm. Four hundred microliters of the homogenate was transferred to a new tube, and 400 µL of fixing solution (6 M guanidine thiocyanate, 20 mM Tris-HCl, and pH 8) was added. The sample was incubated for 10 min at 95°C, and afterward, 400 µL of 2-propanol was added and loaded onto a silica column. The sample was passed through the column for 1 min at 13,000 rpm, and the DNA was adsorbed to the silica column. The column was then washed twice with the washing solution (20 mM NaCl, 2 mM Tris-HCl, pH 8; 80% vol/vol ethanol). The silica column was dried by centrifugation. The DNA was eluted with 100 µL of deionized, sterile, nuclease-free water (pH 7) and kept at –20°C until use.

Real-Time PCR

Real-time PCR primers used in the study and their references are shown in Table 1. For all reactions, Roche Light Cyclers Nano (Roche Diagnostics, Mannheim, Germany) instrument and Biospeedy EvaGreen qPCR Pre-Mix (Bioeksan, Turkey) were used. The reactions contained 1.5 mM MgCl₂, 0.2 mM dNTP mix, 1× reaction buffer, 0.1 U of Fast Start Taq DNA Polymerase, 1× EvaGreen, 5 ng/µL DNA template, and 0.5 µM of each primer. The following thermal cycle program

was applied for all reactions: 95°C for 5 min; 45 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 40 s. To ensure and detect whether if the expected product is amplified during qPCR, melting curve analyzes were applied between 60 and 95°C. The data were analyzed using Roche LightCycler NanoSoftware 1.0. Samples with threshold cycle (C_t) under 40 and with a melting temperature (T_m) between the expected values were reported as positive. Sterile distilled water was used as a template for negative control. Positive control was the 16S rRNA amplifying primers, which was reported by Lane (1991) to amplify 16S rRNA gene region in 99.8% of bacterial species. Babo_VF/R real-time quantitative (q)PCR products were purified by ethanol precipitation and sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Discrimination of S19 Strains from Virulent Strains

For samples that were negative by bacteriological analyses and positive by qPCR, we developed a novel qPCR-based method to differentiate the virulent *B. abortus* strains and the *B. abortus* S19 vaccine strain. We designed qPCR primers (Babo_VF/R, Table 1) targeting the outer membrane protein of *B. abortus*. In silico PCR results using Primer Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) on all DNA sequences registered in the GenBank database are as follows: *Brucella abortus* S19 yielded a 1,132-bp PCR product; *Brucella melitensis* biovar Abortus 2308: yielded a 2,827-bp product; *Brucella abortus* biovar 1 str. 9–941: yielded a 2,827-bp product; and *Brucella abortus* A13334: yielded a 2,488-bp product.

Statistical Analysis

Means and standard deviations of C_t and T_m values from the triplicate samples were calculated using Minitab 17 software (Minitab Ltd., Coventry, UK).

Because the bacterial amounts were not interpreted, the differences in the C_t values were not evaluated in terms of statistical significance. The qPCR products from the Babo_VF/R primer set (Table 1) from the virulent and vaccine *B. abortus* strains were compared in terms of T_m values. Because the minimum difference between the T_m values (6.5°C) was more than 10 times higher than the maximum standard deviation (0.4), the statistical significance of the T_m differences was not tested.

RESULTS AND DISCUSSION

Bacteriological analyses and qPCR experiments were applied to all samples. All samples gave positive results with universal bacterial primers, indicating successful bacterial DNA isolation and amplification (Table 2). All of the qPCR reactions targeting *Brucella ovis* and *B. melitensis* were negative, showing that none of the samples contained *B. ovis* or *B. melitensis*. Results of the *B. abortus* targeted qPCR experiment are given in Table 2. *Brucella abortus*-targeted qPCR was positive for samples 3, 5, 13, 18, 19, 23–26, and 28–34. The results indicated that only samples 29 and 30 were also positive by conventional bacteriological analysis methods.

In silico PCR results indicated successful discrimination of the S19 vaccine strain from virulent strains. For this reason, we applied real-time PCR to DNA of samples 3, 5, 13, 18, 19, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, and 34, and the results are given in Figure 1 and Table 3.

Amplification of the target DNA was observed for all qPCR reactions. The PCR product melting tempera-

tures of the bacteriologically positive samples (89.5 and 90°C) were very different from those of negative samples (82 and 82.5°C). Agarose gel electrophoresis of PCR products revealed that all products were sized between 1,000 and 1,500 bp, except samples 29 and 30, which had product sizes of 2,000 to 2,500 bp. All of the PCR products were sequenced, and BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) analyses of the sequencing results revealed that the 2,000- to 2,500-bp PCR products matched 100% with the virulent *B. abortus* strain DNA (GenBank accession no: CP009625.1), whereas the 1,000- to 1,500-bp PCR products matched 100% with the *B. abortus* S19 vaccine strain DNA (GenBank accession no: CP000887.1). Thus, the *B. abortus* S19 PCR product is 1,132 bp with a T_m between 82 and 82.5°C, and the virulent *B. abortus* PCR product is 2,488 bp with a T_m between 89.5 and 90°C. The virulent *B. abortus* was positive only for samples 29 and 30.

Crasta et al. (2008) stated that the *B. abortus* S19 genome contains DNA regions other than those of the virulent *B. abortus* strains. They reported the first genome of an attenuated live vaccine strain. The researchers determined the complete genome of the vaccine strain S19 compared with that of the virulent strain.

Sangari et al. (1994) described a PCR assay that can distinguish S19 from wild-type *B. abortus*; however, their assay is unable to identify the wild-type *Brucella* species. Amin et al. (2012) used IS711 and *B. abortus* primers, which were used to amplify the *B. abortus* vaccine strain as a positive control and blood samples of vaccinated animals. They reported that the applied PCR was able to differentiate S19-vaccinated animals from infected animals by the size of the PCR product in agarose gel. In another study, Celebi and Otlu (2011)

Table 2. Total bacteria and *Brucella abortus* targeted real-time quantitative PCR threshold cycles (C_t) and melting temperatures (T_m) of positive samples

Sample	Bacteria		<i>B. abortus</i>	
	T_m	C_t	T_m	C_t
3	84.04 ± 0.08	17.29 ± 0.28	81.03 ± 0.08	38.92 ± 0.41
5	84.38 ± 0.17	17.74 ± 0.37	81.11 ± 0.09	39.73 ± 0.51
13	83.68 ± 0.13	17.73 ± 0.13	80.48 ± 0.12	38.37 ± 0.13
18	84.39 ± 0.15	18.35 ± 0.18	80.85 ± 0.02	32.79 ± 0.23
19	83.77 ± 0.07	16.84 ± 0.14	80.65 ± 0.08	37.33 ± 0.18
23	84.02 ± 0.07	16.69 ± 0.29	80.87 ± 0.07	35.34 ± 0.16
24	84.43 ± 0.09	16.83 ± 0.24	80.77 ± 0.05	34.80 ± 0.13
25	84.75 ± 0.02	17.50 ± 0.31	80.49 ± 0.07	36.71 ± 0.17
26	83.57 ± 0.07	17.21 ± 0.18	80.47 ± 0.07	33.62 ± 0.32
28	83.65 ± 0.03	19.44 ± 0.17	80.48 ± 0.09	33.69 ± 0.39
29	84.54 ± 0.07	16.47 ± 0.26	80.48 ± 0.09	34.15 ± 0.22
30	84.55 ± 0.06	25.60 ± 0.33	80.48 ± 0.03	34.75 ± 0.41
31	83.26 ± 0.08	20.13 ± 0.32	80.48 ± 0.04	35.81 ± 0.11
32	84.43 ± 0.08	17.65 ± 0.38	80.48 ± 0.02	34.60 ± 0.33
33	84.12 ± 0.13	17.06 ± 0.26	80.48 ± 0.07	38.44 ± 0.36
34	83.78 ± 0.05	18.33 ± 0.12	80.48 ± 0.11	38.22 ± 0.19

Figure 1. Real-time PCR results. The left-hand panel illustrates the amplifications curves, including samples 29 and 30; the horizontal line indicates the threshold. The distinctive melting peaks of PCR products from samples that were negative by bacteriological analyses and positive by real-time quantitative PCR (samples 29 and 30) are indicated in the right-hand panel. RFU = relative fluorescence unit. Color version available online.

reported the presence of the *ery* gene (904 bp) and the *bp26* gene (738 bp) in the wild strain in milk and vaginal fluid samples. Sangari et al. (1994) reported a PCR assay, AMOS, that could differentiate *B. melitensis*, *B. abortus*, and *B. suis*, and Ewalt and Bricker (2000) modified the assay to differentiate the S19 and RB51 vaccine strains from the wild strain.

Sharifi-Yazdi et al. (2008) used *Brucella* reference, wild, and vaccine strains and developed a multiplex PCR assay to differentiate the 2 vaccine strains from the wild type by using different sizes of DNA fragments.

Table 3. Threshold cycles (C_t) and melting temperatures (T_m) of real-time quantitative PCR targeting the outer membrane protein of *Brucella abortus* to differentiate the virulent and the vaccine strains

Sample	T_m	C_t
3	82.5 ± 0.1	26.95 ± 0.32
5	82 ± 0.2	27.30 ± 0.43
13	82 ± 0.1	25.46 ± 0.49
18	82 ± 0.1	27.55 ± 0.37
19	82 ± 0.4	24.41 ± 0.28
23	82.5 ± 0.1	23.91 ± 0.21
24	82 ± 0.3	26.27 ± 0.25
25	82.5 ± 0.4	27.37 ± 0.23
26	82.5 ± 0.3	21.5 ± 0.41
28	83 ± 0.2	27.55 ± 0.51
29	89.5 ± 0.1	25.15 ± 0.31
30	90 ± 0.2	28.52 ± 0.31
31	82 ± 0.3	26.96 ± 0.32
32	82 ± 0.2	28.46 ± 0.24
33	82 ± 0.1	26.19 ± 0.33
34	82 ± 0.2	22.74 ± 0.25

They reported that *wbo1* and *wbo2* primers produced a ~1,300-bp fragment from RB51 and ~400-bp fragment from other field strains. Primers *eri1* and *eri2* amplified a 178-bp fragment from the field strains and RB51 genomic DNA, but no fragments from that of the S19 strain (Sharifi-Yazdi et al., 2008). Nan et al. (2014) reported a duplex PCR that can identify *B. suis* S2 and biovar 1 strains of *B. suis*.

Nevertheless, no qPCR-based technique has been available for discriminating virulent strains from the *B. abortus* S19 vaccine strain. Herein, we described a qPCR method that can differentiate the virulent *B. abortus* strain from the *B. abortus* vaccine strain by differences in T_m of the qPCR products.

We performed sampling in the Thrace region because (1) many dairy companies supply their milk from the cows produced in this region, (2) the region remains endemoepidemic for brucellosis, (3) the region is the pilot region for vaccination of animals for brucellosis, and (4) the traditional vaccination route was changed in 2012 and live-attenuated vaccine has been administered through the conjunctiva since then (Republic of Turkey Ministry of Food, Agriculture and Livestock, 2012).

In studies screening for *Brucella* in milk, bacterial culture yields a very valuable result. However, it is difficult to isolate *Brucella* spp., especially in their first isolation (Kaynak-Onurdag et al., 2008). After isolation, the diagnostic criteria are subjective and need qualified laboratory personnel to differentiate bacteria. Also, *Brucella* is a potential biological hazard for personnel

(Sharifi-Yazdi et al., 2008). For these reasons, detecting *Brucella* spp. DNA, whether for diagnosis or screening, is a common method used by researchers (Hamdy and Amin, 2002; Xiao et al., 2005; O'Leary et al., 2006; Arasoglu et al., 2013; Alamian et al., 2014; Wareth et al., 2014). But, as shown in our study, under conditions in which vaccination and control of vaccination are done regularly, the live attenuated vaccine strain may cause false-positive PCR results.

The increase in the number of positive samples by PCR is associated with the sensitivity of the method, and differentiating the virulent and vaccine strain has been disregarded. However, the vaccine strain may be detected in milk, especially during the estrus periods of the animals (Barbosa et al., 2014).

In our study, we determined that the greater number of positive results detected by PCR, while *Brucella* spp. was isolated in only 2 samples, is explained by the presence of the vaccine strain, causing false-positive PCR. As a result, we can report that PCR can be used for screening *Brucella* spp. in milk samples, but differentiating the virulent and vaccine strain is important. False-positive results may lead to economic losses and to the perception that brucellosis remains in that region.

In this study, 2.02% of the samples were positive for *Brucella* when tested by both the bacteriological method and PCR. We concluded that, to obtain true positive results in *Brucella* spp. screening studies for milk, differentiating the virulent and vaccine strain must be considered.

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