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RESEARCH ARTICLE

Detection of *Brucella* Antibody and DNA in Cow Milk by ELISA and PCR Methods

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Summary

The aim of the present study was to determine comparatively the presence of anti-*Brucella* antibody and *Brucella* DNA in cow milk. Anti-*Brucella* antibody was detected by ELISA based on the lipopolysaccharide (LPS) as diagnostic antigen. Besides, the presence of *Brucella* DNA in milk samples was screened by *ery*CD gene-targeted PCR and *B. abortus* DNA was determined by amplification of *alk*B genes. For this purpose, 70 raw cow milk samples collected from open markets were used. Among these samples, 15 samples (21.4%) were found positive for anti-*Brucella* LPS antibody in ELISA. In contrast, only 5 milk samples (7.1%) were determined as positive by *ery*CD gene-targeted PCR. All of the *ery*CD positive samples giving an amplicon of 904 bp indicated the presence of wild-type Brucella DNA but not *B. abortus* S19 vaccine strain allowing amplification of only an amplicon of 202 bp. In addition, amplification of the *alk*B gene demonstrated the presence of *B. abortus* DNA in 5 *ery*CD positive samples. No statistical agreement was observed between ELISA and PCR results with 95% confidence interval. These results strongly suggest that use of both ELISA and PCR methods could lead to more reliable diagnosis of brucellosis from bovine milk samples.

Keywords: *Brucella, Bovine, Milk, ELISA, PCR*

İnek Sütünde *Brucella* Antikoru ve DNA'sının ELISA ve PCR Yöntemleri ile Tespiti

Özet

Bu çalışmada inek sütünde anti-*Brucella* antikorları ve *Brucella* DNA'sının karşılaştırmalı yöntemlerle belirlenmesi amaçlanmıştır. Anti-*Brucella* antikorların tespitinde spesifik tanı antijeni olarak lipopolisakkaride (LPS) dayalı ELISA kullanıldı. Ayrıca, süt örneklerinde *Brucella* DNA varlığı *ery* genine dayalı PCR tekniği ile araştırıldı ve *B. abortus* DNA varlığı ise *alk*B geni amplifikasyonu ile belirlendi. Bu amaçla, 70 adet çiğ inek sütü örneği yerel pazarlardan toplandı. Bu örneklerin 15'i (% 21.4) anti-*Brucella* LPS antikoru yönünden ELISA'da pozitif bulundu. Buna karşın, 5 süt örneğinin (% 7.1) eryCD genine dayalı PCR'da pozitif olduğu belirlendi. Tüm *ery*CD pozitif örneklerin 904 bp boyutunda amplikon vermesi, *Brucella* DNA'sının saha suşuna ait olduğunu ancak yalnızca 202 bp büyüklüğünde amplikon çoğaltılabilenn *B. abortus* S19 aşı suşuna ait olmadığını gösterdi. Ayrıca, *alk*B geninin PCR'da çoğaltılması ile *ery*CD pozitif bulunan 5 örnekte de *B. abortus* DNA varlığı belirlendi. %95 güven aralığında, PCR ve ELISA sonuçları arasında istatistiksel uyumluluk gözlenmedi. Bu bulgular, inek süt örneklerinde brusellozis tanısının ELISA ve PCR yöntemlerinin birlikte kullanımı ile daha güvenilir olabileceğine kuvvetle işaret etmektedir.

Anahtar sözcükler: *Brucella, Sığır, Süt, ELISA, PCR*

INTRODUCTION

Brucellosis is a widespread zoonotic disease causing considerable economic losses in ruminants and transmission of the pathogenic *Brucella* strains to humans occurs as a result of consuming contaminated milk and milk products and direct contact with the infected animals [1-3]. Thus, the determination of the infected

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animals is of paramount importance for public and animal health. In dairy cattle, bacteriological, serological and molecular methods have been carried out for the diagnosis of brucellosis [4-6]. Although isolation of the bacteria leads to the definitive diagnosis of the disease, bovine brucellosis diagnosis is essentially based on the serological methods using serum or milk samples [4-6]. As milk particularly reflects IgG based antibody response of the animal [7,8] and is a non-invasive sampling method, its use instead of blood in serological detection represents an important advantage in lactating animals.

Milk Ring Test (MRT) has been used as a screening test for bovine brucellosis, and is an adaptation of the agglutination test performed with the milk [9]. MRT has relatively low sensitivity and leads to wrong interpretations with colostrums, milk at the end of lactation period and milk from cow with mastitis [6]. Its specificity, however, is doubtful when *Brucella* prevalence is low [10]. Milk ELISA was found more sensitive and specific than MRT in detecting anti-*Brucella* antibodies in milk [4,11].

In recent years, detection of *Brucella* DNA by PCR-based methods in milk samples have been developed [12-14]. These methods are rapid and accurate and allow testing a great number of samples to detect the presence of the pathogens [15]. A number of nucleic acid sequences have been targeted for the development of *Brucella* genus-specific PCR assays, including 16S rRNA, 16S-23S intergenic spacer region, *omp*2, *bcsp*31 [13,16]. In addition, *IS711* element downstream of the *alk*B gene allows the detection of *B. abortus* and also *ery*CD gene regions facilitate the discrimination of *B. abortus* field strains from the S19 vaccine strain [17-20].

The aim of the present study was to detect comparatively the presence of anti-*Brucella* antibody and *Brucella* DNA in cow milk. For these purposes, our previously described LPS-based ELISA method was adapted to ELISA to detect anti-*Brucella* antibody in milk [21]. Furthermore, eryCD and *alk*B gene-based PCR assays using milk DNA as template were developed and used for determination of the presence of *Brucella spp*. and *B.abortus* and also discrimination of *B. abortus* field strains from the vaccine strain S19.

MATERIAL and METHODS

Milk Samples and Milk Whey Preparation

A total of 70 milk samples from cows with unknown brucellosis status were collected from 8 different open markets in Samsun (Turkey) between January-March 2008. Seven milk samples from *B. abortus* isolated animals and 16 samples from *B. abortus* S19 vaccinated cattle were collected. Forty five milk samples from non-vaccinated cows evaluated as negative with MRT and whey agglutination tests as described in a previous study [22] were used as negative controls. Milk whey was prepared by the addition of 200 µl of commercial liquid rennet (strength 1: 10000; 0.3%, w/v) to 10 ml of each milk sample and incubation at 37°C for 30 min until the coagulation occurred [23]. Following removal of the casein by filtration and centrifugation, clear milk whey was collected and stored at -20°C until use.

B. abortus Strain, Culture, Enumeration and LPS Preparation

Brucella abortus S19 vaccine strain supplied from Veterinary Research and Control Institute (Pendik, İstanbul) was grown at 37°C for 3 days on blood agar base (Merck, Germany). A single smooth colony selected by morphology and acriflavine agglutination was isolated and cultured in Brain Heart Infusion Broth (BHI, Oxoid, Cambridge, UK). Colony count was determined by inoculation of 0.1 ml suspensions from ten-fold serial dilutions onto BHI agar plates and incubation at 37°C for 24 h.

To prepare *B. abortus* LPS as an immunodominant antigen, fresh *Brucella* culture was inactivated by formaldehyde (0.5%) and stored at 4°C for 2 days. Inactivated cells were harvested by centrifugation, washed three times in physiological saline solution and LPS was extracted by hot phenol-water method as previously described by Caroff et al.[24].

ELISA Procedure

Indirect ELISA method previously described by Genç et al.[21] was adapted to test milk whey. Briefly, micro-plates were coated with 100 µl of 5 µg/ml of *B. abortus* crude LPS prepared in carbonate buffer (0.1 M, pH 9.6) and kept overnight at +4°C. The microwells blocked using 200 µl/well of 1% fish gelatine in phosphate buffered saline (PBS, Sigma Aldrich, St-Louis, USA) containing 0.1% Tween 20 (FG-PBST) were incubated at 37°C for 1 h. One hundred microliters of milk whey samples diluted 1:5 in FG-PBST were added to microwells. Alkaline phosphatase conjugated rabbit anti-bovine IgG (Sigma-Aldrich, St-Louis, USA) diluted 1:30 000 were added and incubated for 1 h at 37°C. After addition of 100 µl of pNPP (p-Nitrophenyl Phosphate, Sigma-Aldrich) as substrate, and incubation for 1 h at 37°C, the absorbance was read at 405 nm in ELISA reader (Digital Analog Systems, DAS RS 232, Rome, Italy). Each assay was carried out in duplicate.

DNA Extraction

Total milk DNA was obtained with phenol-

chloroform-isoamyl alcohol extraction method as described by Leal-Klevazas et al.[25]. Briefly, 400 µl of lysis solution (2% Triton X-100, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0]) and 10 µl of proteinase K (10 mg/ml) were added to 400 µl of samples taken from the cream layer of each milk. Following centrifugation, the pellet was rinsed with 1 ml of 70% ethanol, dried and resuspended in 20 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1mM disodium EDTA). Concentration and purity of DNA were measured by absorbance at 260 and 280 nm wavelengths using a UV spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK) and stored at -20°C until use.

PCR Assays

Primer sequences of *ery*CD and *alk*B genes and PCR assay conditions were given in *Table 1.* PCR was carried out in a total volume of 50 µl, using 1xPCR buffer, 200 ng of purified genomic DNA, 20 pmol of each oligo-nucleotide primer, 2 mM $MgCl_2$, 200 mM of each dNTP and 2.5 U of Taq DNA polymerase (MBI Fermentas, Germany). To assess the detection limit of the PCR assay, ten-fold dilutions of *B. abortus* S19 vaccine strain in PBS ranging from 1.0 x 10^9 to 1.0 x 10^1 CFU ml^{-1} was inoculated into *Brucella* negative milk samples. Total DNA extraction and determination of its concentration were carried out and PCR assays were performed as described above. The reactions were performed with a DNA thermocycler (Biometra-Tpersonal, Göttingen, Germany). PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel in 1xTBE buffer, pH 8.0 and visualized under ultraviolet light after staining with ethidium bromide. A Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas) was used as a DNA size marker.

Statistical Analysis

All statistical analyses were performed at 95% confidence interval (CI) by using Win Episcope version 2.0 programme. The cut-off point, sensitivity and specificity of ELISA were determined using receiver-operating characteristic (ROC) analysis. ELISA and PCR results were compared by test agreement analysis and evaluated on the basis of kappa (κ) value.

RESULTS

Detection of Anti-Brucella Antibody in Milk by ELISA

The cut off value of LPS-based ELISA was determined as 0.500 at OD405 by ROC analysis on the basis of the results obtained from 23 positive and 45 negative pre-evaluated milk whey samples described in details in material and methods. The sensitivity and specificity of the test at 95% confidence interval was found 87% and 100%, respectively. Positive and negative predictive values (PPV and NPV) were detected as 100% and 93.75%, respectively. Area under curve (AUC) being found 92.42% by ROC analysis demonstrates high diagnostic performance of ELISA, particularly for its specificity. When 70 cow milk whey samples were tested with ELISA, 15 samples (21.4%) were found positive and 55 samples (78.6%) were negative *(Table 2).*

PCR Analysis for Detecting Brucella eryCD and alkB Genes

PCR amplification of *ery*CD gene region was performed and only an amplification of 904 bp fragment was detected from 5 (7.1%) out of 70 milk samples *(Fig. 1).* These *ery*CD amplicon positive samples were further analyzed by *alk*B gene-targeted PCR to determine *Brucella* species and all of these samples were confirmed as *B. abortus* by amplification of a 136 bp DNA fragment *(Fig. 2).* All positive milk samples contained only wild-type *B. abortus* DNA but not *B. abortus* S19 vaccine strain DNA, giving only a 202 bp-amplicon *(Fig. 1 lane 3).*

Determination of Detection Limit of PCR

PCR detection limit was determined as 1.0 x 10^3 CFU ml^{-1} *Brucella* DNA from artificially contaminated negative milk samples by *ery*CD-targeted PCR.

Table 1. *PCR conditions and primers for eryCD and alkB genes*
Tablo 1. *eryCD ve alkB genleri için PCR koşulları ve primerler*

Cyling Parameters	*eryCD gene*		*alkB gene*	
	Temperature	**Duration**	**Temperature**	**Duration**
Initial denaturation	95°C	10 min	95°C	10 min
Denaturation	94°C	30 sec	94°C	15 sec
Annealing	60°C	30 sec	57°C	1 min
Extention	72°C	1 min and 40 sec	72°C	1 min and 40 sec
Final extention	72°C	10 min	72°C	10 min
	40 cycles		45 cycles	
Primer (F)	5′-GATCGCCATCGACTGCTGGG-3′		5′-GCGGCTTTTCTATCACGGTATTC-3′	
Primer (R)	5′-GGTCATCGGCATCGGCCATGGC -3′		5′-CATGCGCTATGATCTGGTTACG-3′	

Table 2. *Comparative analysis between ELISA and PCR results*
Tablo 2. *ELISA ve PCR sonuçlarının karşılaştırmalı analizi*

PCR Results	ELISA Results		Test Agreement
	Positive (n=15)	Negative (n=55)	
Positive (n=5)	1	4	κ= -0.008
Negative (n=65)	14	51	(-0.202/+0.186)

(*) Values in parenthesis indicate lower and upper kappa values

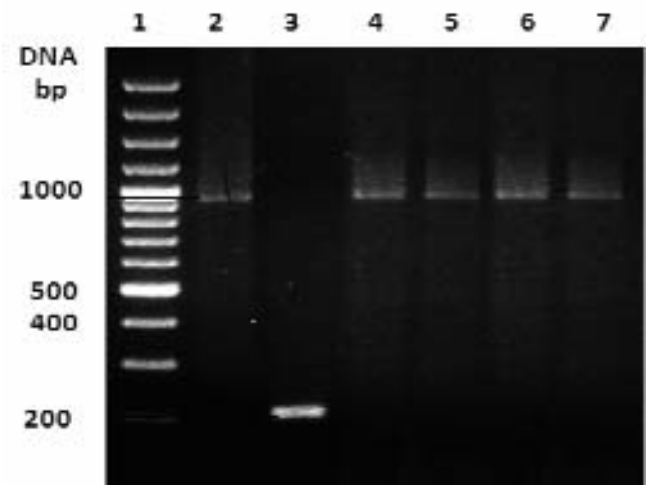

Fig 1. Gel electrophoresis of *Brucella ery*CD genes region amplicon from total DNA of cow milk. **Lane 1:** 100 bp DNA ladder; **Lane 2:** *B. abortus* field strain (904 bp); **Lane 3:** *B. abortus* S19 vaccine strain (202 bp); **Lane 4-7:** positive milk samples

Şekil 1. İnek sütü total DNA'sından çoğaltılan *Brucella ery*CD gen bölgesi jel elektroforezi. **Sütun 1:** 100 bp DNA belirteci; **Sütun 2:** *B. abortus* saha suşu (904 bp); **Sütun 3:** *B. abortus* S19 aşı suşu (202 bp); **Sütun 4-7:** pozitif süt örnekleri

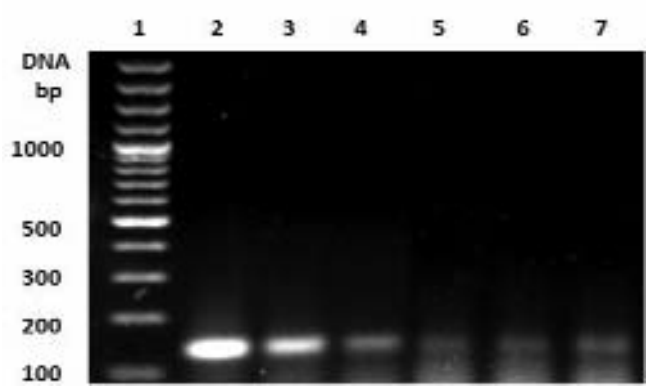

Fig 2. Gel electrophoresis of *B. abortus alk*B gene amplicon from total DNA of cow milk. **Lane 1:** 100 bp DNA ladder; **Lane 2:** *B. abortus* field strain (136 bp); **Lane 3-7:** positive milk samples

Şekil 2. İnek sütü total DNA'sından çoğaltılan *B. abortus alk*B geni jel elektroforezi. **Sütun 1:** 100 bp DNA belirteci; **Sütun 2:** *B. abortus* saha suşu (136 bp); **Sütun 3-7:** pozitif süt örnekleri

DISCUSSION

Serological tests such as MRT and ELISA are widely used for the detection of anti-*Brucella* antibody in milk [7,26,27]. However, MRT often causes wrong results [5,6] and its sensitivity and specificity have been found lower than ELISA methods [7,26,28]. Because of accuracy and less cross-reactions in determining *Brucella* antibody in milk and serum, LPS-based ELISA is preferentially used [4,27]. We adapted a previously described LPS-based ELISA [21] to milk antibody detection and determined its cut-off value as 0.500 at OD_{405} by ROC analysis in order to obtain a specificity of 100%. Although its sensitivity decreased to 87%, percentage of *Brucella* antibody positivity of the milk samples tested in this study was found 21% and considered as high for Samsun region, where vaccination against brucellosis is rarely applied.

Based on these results, PCR amplification targeting the genus and species-specific genes, *ery*CD and *alk*B, was performed to determine and confirm the presence of *Brucella* DNA in milk samples. *Brucella ery*CD gene-targeted PCR was carried out not only for detecting *Brucella spp.* DNA in milk samples but also for discriminating DNA of wild-type *B. abortus*, from S19 vaccine strain DNA as described in earlier studies [12,13,18]. In this study, amplification of a 904 bp fragment by *ery*CD gene-targeted PCR demonstrated the presence of the wild-type *Brucella* DNA in 5 milk samples. Detection of an amplicon of 136 bp by *alk*B gene-targeted PCR from the same samples allowed, however, identifying the presence of *B. abortus* DNA. Furthermore, no amplification of a 202 bp fragment by *ery*CD gene-targeted PCR confirmed only the presence of *B. abortus* wild-type strain in the same samples but not the S19 vaccine strain containing a deletion of 702 bp. From these results, it can be proposed that *ery*CD in follows *alk*B genes-targeted PCRs would be used as screening and differentiating molecular diagnostic tests for investigation of brucellosis status of the animal from milk.

When PCR results were compared with ELISA results, 51 milk samples were detected as negative with both tests *(Table 2).* While 15 samples were found positive in ELISA, only one sample of them was detected as positive by PCR. In the same manner, among 5 PCR positive samples only one sample was detected as positive in ELISA. As seen in *Table 2,* statistical analysis showed no agreement between both ELISA and PCR test results because the kappa value was significantly lower than 1. Our findings were similar to that of Romero et al.[12] reporting that 7 PCR negative samples were found positive in ELISA, and only one ELISA negative sample was detected as positive by PCR. This difference can be

due to i) long term persistence of *anti-Brucella* antibody without presence of the disease agent in milk or ii) relatively low detection limit of PCR. Because detection limit of eryCD gene-targeted PCR was determined as 1.0 x 10^3 CFU ml^{-1}, it is possible that some milk samples containing bacteria less than the detection limit failed to be found as positive. On the other hand, no detection of *Brucella* DNA by PCR in majority of the ELISA positive samples could be explained by having samples from animals in their chronic phase of the disease.

The consumption of contaminated milk and milk products is one of the main transmission ways of pathogenic *Brucella* strains to humans. For that reason, fast and accurate evaluation of brucellosis status of the milk and its products is paramount for public health. In conclusion, as significantly different results were obtained by the detection of anti-*Brucella* antibody and *Brucella* DNA in cow milk, the present study suggests that accurate evaluation of brucellosis status of cow milk and discrimination of *B. abortus* field strains from the vaccine strain S19 would be assured by simultaneous use of both ELISA and PCR assays.

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