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ORIGINAL ARTICLE

# Detection of *Brucella melitensis* DNA in the milk of sheep after abortion by PCR assay[#]

## Detección de ADN de *Brucella melitensis* mediante la prueba de PCR en muestras de leche de ovejas postaborto

**Z Ilhan[*], H Solmaz, A Aksakal, T Gulhan, IH Ekin, B Boynukara**
University of Yüzüncü Yıl, Faculty of Veterinary Science, Department of Microbiology, 65080 Van, Turkey

**RESUMEN**

El diagnóstico de laboratorio de brucelosis es generalmente realizado por métodos microbiológicos y serológicos. La prueba PCR es reconocida como una alternativa específica y sensible para la detección de diferentes agentes bacterianos. Se realizó una evaluación de la prueba PCR para la detección de ADN de *Brucella melitensis* en leche de oveja. Ciento dos muestras tomadas de ovejas postaborto fueron analizadas por métodos de cultivo bacteriológico, prueba PCR y prueba del anillo en leche (MRT). El PCR detectó ADN de *B. melitensis* en 24 (23,5%) de 102 muestras de leche, mientras que solamente 8 (7,8%) muestras de leche fueron positivas a *B. melitensis* por cultivo directo. El MRT detectó 28 (27,4%) muestras positivas de leche. El límite de detección de *B. melitensis* 16 M por PCR fue de $1,7x10^3$-$1,7x10^4$ ufc/ml en leche inoculada. La concordancia entre PCR y MRT fue 96%. La sensibilidad diagnóstica y la precisión fueron determinadas como el 100% y el 81,3% respectivamente para la prueba de PCR y el 75% y el 75% para MRT. En conclusión, PCR es una herramienta muy útil para el diagnóstico rápido de *B. melitensis* en leche de oveja.

*Key words*: *Brucella melitensis,* sheep, milk, PCR.

*Palabras clave*: *Brucella melitensis*, oveja, leche, PCR.

## INTRODUCTION

Brucellosis continues to be a problem for animal and humans throughout the world (Bricker 2002). The genus of *Brucella* comprises of Gram-negative, non-motile and facultative intracellular pathogens and six species are recognized within the genus: *Brucella melitensis, B. abortus, B. ovis, B. canis, B. suis* and *B. neotomae* (Moreno *et al* 2002, Rivers *et al* 2006). With respect to the current classification of *Brucella* species according to the preferential host, two new species have been recognized, *B. pinnipediae* (for pinniped isolates) and *B. cetaceae* (for cetacean isolates) (Cloeckaert *et al* 2001). *B. melitensis* causes an important infectious disease affecting mostly small ruminants and humans (Blasco *et al* 1994). The udder is an important predilection site for *Brucella.* In dairy animals, microorganisms localize in the supramammary lymph nodes and these can continue to excrete them in milk (Cordes and Carter 1979, Refai 2003). People contract the disease by direct contact with contaminated fetal membranes or, more commonly, as a result of the consumption of contaminated unpasteurized milk and cheese products (Young 1983, Wallach *et al* 1994). Studies performed in different regions of Turkey have shown that *B. melitensis* is responsible for approximately 20% of the abortion cases in sheep (Arda *et al* 1987, Kenar *et al* 1990). Thus, brucellosis is one of the major problems of the sheep industry in Turkey, and it could be estimated that the disease results in huge economic losses. Moreover, in the region of Van in East Anatolia in Turkey, humans often consume Herby Cheese (O*tlu Peynir*) produced from raw milk (Ekici *et al* 2006).

The methods for the diagnosis of brucellosis comprise tests for isolation and identification, tests for detection and estimation of antibodies induced in response to the agent, as well as an allergic test, brucellin, particularly in small ruminants (Refai 2003). Serologic methods are not conclusive, because not all infected animals produce detectable levels of antibodies and because cross-reactions with antigens other than those from *Brucella* can give false-positive results. However, milk ring test (MRT) is probably the most widely used test for screening and monitoring of brucellosis in dairy cattle (Alton *et al* 1988). The sensitivity of the bacteriological culture methods depends on the viability and numbers of *Brucella* in the sample, and the nature of the sample which is commonly contaminated with other bacteria. Thus, culture methods are not always successful as they are time-consuming and the handling of microorganism is hazardous (Refai 2003). PCR has the potential to meet the need for better diagnostic tools for several infectious diseases caused by fastidious or slow growing bacteria (Romero *et al* 1995, Bricker 2002). Although previous

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[*] Faculty of Veterinary Science, Department of Microbiology, 65080 Van, Turkey; zilhan@yyu.edu.tr, ziyavan52@yahoo.com

studies have demonstrated that PCR based assays can be used to detect *Brucella* DNA in pure cultures (Romero *et al* 1995, Rijpens *et al* 1996) and different samples of goats (Leal-Klevezas *et al* 1995, Leal-Klevezas *et al* 2000) and bovine (Romero and Lopez-Goñi 1999, O'Leary *et al* 2006), the brucellosis of the sheep has received comparatively little attention by using milk as material. The purpose of this study was to investigate the viability of the PCR assay as a diagnostic tool for the detection of *B. melitensis* DNA in the milk of sheep after abortion and to compare its results with traditional bacteriological culture methods and MRT.

## MATERIAL AND METHODS

### SAMPLING, BACTERIOLOGICAL EXAMINATIONS AND MRT

During 2 successive lambing seasons (in the years of 2004-2005, 2005-2006) 102 sheep milk samples after abortion in 92 different sheep flocks were collected in the Van region of Turkey. The total sheep number was 10.833 and 1.263 ewes aborted in these flocks. The materials used in this study were collected from animals that were not previously vaccinated against *Brucella*. All samples were taken approximately 1-3 days after abortion. Samples were collected under sterile hygienic conditions from both udders. Fifteen ml of the milk were sampled and subdivided into 5 ml aliquots. These were used in culture, PCR and MRT. Samples destined for PCR assay were kept at –20 °C until processing.

Swabs from the fatty top layer and centrifugation pellet (3,000 rpm for 15 min at 4 °C) of raw milk was previously tested onto duplicate plates with blood agar (Merck, Darmstadt, Germany) containing 5% defibrinated sheep blood, blood agar base Nº 2 (Oxoid, Hampshire, England) supplemented with *Brucella* selective supplement (Oxoid, SR083A) and 5% defibrinated sheep blood. The defibrinated sheep blood was collected from *Brucella*-free sheep from the Research Farm of the Faculty of Veterinary Science, University of Yüzüncü Yıl. The samples were also plated onto Farrell's modified serum dextrose agar. The latter was prepared from blood agar base Nº 2 supplemented with *Brucella* selective supplement (Oxoid, SR083A), 7% horse serum and 1% glucose (Stack *et al* 2002). All plates were incubated at 37 °C, both in air and microaerobically (5-10% $CO_2$) for 5-7 days. Identification and typing of *Brucella* strains were performed using standard classification tests, including growth characteristics, catalase, oxidase and urease activity, $H_2S$ production, growth in presence of thionin (40 µg/ml, 20 µg/ml and 10 µg/ml) and basic fuchsine (20 µg/ml and 10 µg/ml), lysis by Tbilisi (Tb) phage and agglutination with monospecific A and M antisera (Alton *et al* 1988, Quinn *et al* 1994). Identification of the other bacteria was performed by conventional biochemical tests (Quinn *et al* 1994). *B. melitensis* reference strain 16 M, *B. abortus* reference strain S19 (for control of Tb phage) and Tb phage, which had been kept in the culture collection of the Department of Microbiology, Faculty of Veterinary Science, University of Yüzüncü Yıl, were supplied by the Pendik Veterinary Control and Research Institute, İstanbul, Turkey.

### MRT

A modification of the procedure described by Alton *et al* (1988) was used in the MRT. MRT antigen prepared from *B. abortus* S99 strain and stained with tetrazolium chloride was supplied by the Pendik Veterinary Control and Research Institute, İstanbul, Turkey. The test was performed in sterile tubes and the milk samples were tested within 2 h after collection. Fresh milk samples from the individual sheep were thoroughly shaken and 1 ml of the milk was transferred into to a fresh tube and 30 µ1 of MRT antigen was added. The tubes were mixed thoroughly and incubated at 37 °C for 3 h. When the antigen precipitated in the bottom of tubes and/or the purple band occurred at the top of milk, these samples were then regarded as positive (Türütoğlu *et al* 2003).

### DNA EXTRACTION AND PCR

*B. melitensis* DNA was extracted from milk by the method of Leal-Klevazas *et al* (2000). Briefly, frozen milk samples were thawed at room temperature and 400 µl of lysis solution (100 mM Tris-HCl (pH 8), 100 mM NaCl, 1% SDS, 2% Triton-X100) and 10 µl of proteinase K (10 mg/ml) were added to 400 µl of the fatty top layer of each milk sample. The contents were incubated at 50 °C for 30 min. Thereafter, 400 µl of saturated phenol (liquid phenol containing 0.1% 8-hydroxyquinoline, saturated and stabilized with 10 mM Tris-HCl (pH 8) and 0.2 % of 2-mercaptoethanol) were added, mixed thoroughly and centrifuged at 8.000 x g for 5 min. The aqueous layer was transferred to a fresh tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed thoroughly and centrifugated as described above. The upper layer was again transferred to a fresh tube and an equal volume of 7.5 M ammonium acetate was added and mixed thoroughly. The contents were kept on ice for 5 min, centrifugated at 8,000 x *g* for 5 min and the aqueous content was transferred to a fresh tube. Two volumes of 95% ethanol were added, mixed and the tubes were stored at –20 °C for 12 h. DNA was recovered by the final centrifugation as described above, the pellets were rinsed with 1 ml of 70% ethanol, dried and resuspended in 30 µl TE buffer (AppliChem, Darmstadt, Germany). In addition, a commercial DNA extraction kit (Dneasy® Tissue Kit, Qiagen, Hilden, Germany) was also used in this study. For this purpose, 25 µg of the fatty top layer were used as the initial extraction material. Subsequent extraction stages were applied according to the manufacturer's recommen-

dations. Finally the DNA extraction was stored at -20 °C until they were processed in a thermal cycler.

### SYNTHETIC OLIGONUCLEOTIDE DESIGN

The *B. melitensis*-specific primers used were previously described by Bricker and Halling (1994). The sequences of the primers were 5'-AAA, TCG, CGT, CCT, TGC, TGG, TCT, GA-3' (*B. melitensis*-specific primer) and 5'-TGC, CGA, TCA, CTT, AAG, GGC, CTT, CAT-3' (IS711-specific primer).

### DNA AMPLIFICATION AND DETECTION OF PCR PRODUCTS

PCR was carried out in a total volume of 50 µl, using 10 mM Tris-HCl (pH 9), 3 mM $MgCl_2$, 50 mM KCl, 0.1% Triton-X100, 200 mM of the each four deoxynucleotide triphosphates (Lavora, Tellow, Germany), 0.4 mM of each primer (50 pmol), 2 IU of *Taq* polymerase (Fermentas, Opelstrasse 9, Leon-Rot, Germany) and 2 µl template. The amplification was performed in a DNA thermal cycler (Thermo, Px2 Thermal Cycler, USA) as follows: initial denaturation step at 94 °C for 4 min, and 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. The final incubation was at 72 °C for 5 min (Leal-Klevezas *et al* 2000).

Amplification products were resolved in a 1.5% (w/v) agarose gel containing 1xTBE buffer (100 mM Tris-HCl (pH 8), 90 mM boric acid and 1 mM $Na_2EDTA$) and stained with ethidium bromide (0.5 µg/ml) and evaluated by a computerized image analysis system (Spectronics Co., Gl-5000, England). A visible band of appropriate size (731 bp) was considered as a positive reaction for *B. melitensis*. A positive control (based on DNA from *B. melitensis* 16 M) and a negative control (DNases and RNases free water, AppliChem) were included in all the tests. To check the reliability of the results and to detect any external contamination, all samples were processed in duplicate.

### DETERMINATION OF DETECTION LIMIT OF THE PCR IN INOCULATED MILK

*B. melitensis* 16 M strain was grown on trypticase soy agar (TSA, Merck) at 37 °C for 48 h. A single colony was removed from TSA, placed in trypticase soy broth (Merck) and incubated at 37 °C for 48 h. Thereafter, the culture was prepared in sterile saline and 10-fold dilutions (from $10^{-1}$ to $10^{-10}$) were made. From these dilutions, 0.1 ml suspension was inoculated onto TSA plates and incubated at 37 °C for 48 h and the colonies present were then enumerated. The number of organisms in the dilutions was estimated spectro-photometrically at 623 nm and the concentration of the original *B. melitensis* culture was estimated as 1.7 x $10^9$ cfu/ml (OD 0.18). To assess the limit of detection of the PCR assays, 12 raw milk samples collected from *Brucella*-free sheep from the Research Farm of Faculty of Veterinary Science, University of Yüzüncü Yıl, were artificially contaminated with a known decreasing number of pure *B. melitensis* 16 M strain. The final concentrations of the organism in milk was 1.7 x $10^8$, 1.7 x $10^7$, 1.7 x $10^6$, 1.7 x $10^5$, 1.7 x $10^4$, 1.7 x $10^3$, 1.7 x $10^2$ and 1.7 x $10^1$ cfu/ml. *B. melitensis* DNA was extracted from all dilutions of milk, and processed by PCR as described earlier. The final concentrations of organisms were verified by plating onto TSA.

### STATISTICAL ANALYSIS

PCR assay and MRT results were analyzed by comparison of two proportions method (Armitage 1977). In the present study, to calculate diagnostic sensitivity and specificity of the PCR and MRT, 8 milk samples from which *B. melitensis* was isolated were accepted as true positives. The sensitivity and specificity were calculated as described previously (Thrusfield 1986).

## RESULTS

### COMPARISON OF PCR WITH BACTERIOLOGICAL CULTURE AND MRT

A total number of 8 (7.8%) *Brucella* positive samples were detected only in Farrell's modified serum dextrose agar of the 102 milk samples. All *Brucella* organisms were identified and biotyped as *B. melitensis* biovar 3 by tests; positive catalase, oxidase and urease, reduced nitrate, negative $H_2S$, no $CO_2$ requirement, no lysis by Tb phage, growth in the presence of thionin and basic fuchsin and positive agglutination with monospecific A and M antisera. *B.* ovis and the other *Brucella* species were not isolated in the remaining 94 (92.1%) milk samples in this study (table 1). In culture, 21 milk samples were positive for *Staphylococcus* spp. (hemolytic activity on 5% sheep blood agar, colony and microscopic morphology, positive catalase and negative oxidase tests), 14 for *Streptococcus* spp. (hemolytic activity on 5% sheep blood agar, colony and microscopic morphology and negative catalase test), 12 for *E. coli* (positive indole production, methyl red, lactose, maltose, mannitol, mannose and xylose fermentation activities, negative oxidase, Voges-Proscauer, urease and hydrogen sulphide tests), 8 for *S. aureus* (golden-yellow pigment and hemolytic activity on 5% sheep blood agar, positive catalase, coagulase (with rabbit plasma), DNase, mannitol and maltose fermented tests) and 5 for *Corynebacterium* spp. (colony and microscopic morphology, positive or negative catalase, urease, nitrat reductase tests and aesculin hydrolysis). In 24 (23.5%) of the milk samples, PCR products with a molecular size of 731 bp indicative of *B. melitensis* DNA were obtained. Of the 102 milk samples, 28 (27.4%) tested positive by MRT. When culture and PCR results were compared, 8 samples tested

positive and 78 samples tested negative with both methods. All the milk samples that tested positive by culture also tested positive by PCR (table 1). When PCR and MRT results were compared, 22 samples tested positive and 72 negative with both assays. The agreement between PCR and MRT was determined as 96%. When the results of PCR and MRT were compared with the bacterial culture results, the diagnostic sensitivity and specificity were determined as 100% and 81.3% respectively for PCR assay and 75% and 75% for MRT. Table 1 shows the results of bacteriological isolation, PCR assay and MRT.

**Table 1.** Bacteriological culture, PCR assay and MRT results of 102 milk samples taken from sheep after abortion.

Cultivo bacteriológico, prueba PCR y resultados MRT de 102 muestras de leche tomadas de ovejas postaborto.

| Bacteriological culture | | | PCR assay [a] | | MRT [b] | |
|---|---|---|---|---|---|---|
| Milk (n=102) | | | Milk (n=102) | | Milk (n=102) | |
| Blood Agar | Blood Agar Base No:2 | Farrell's Agar | + | - | + | - |
| 0 | 0 | + (n=8) [c] | 8 | 0 | 6 | 2 |
| 0 | 0 | - (n=94) | 16 | 78 | 22 | 72 |
| 0 | 0 | 8 | 24 | 78 | 28 | 74 |

[a] Polymerase chain reaction assay, [b] Milk ring test, [c] All *Brucella* strains were typed as *B. melitensis* biovar 3.

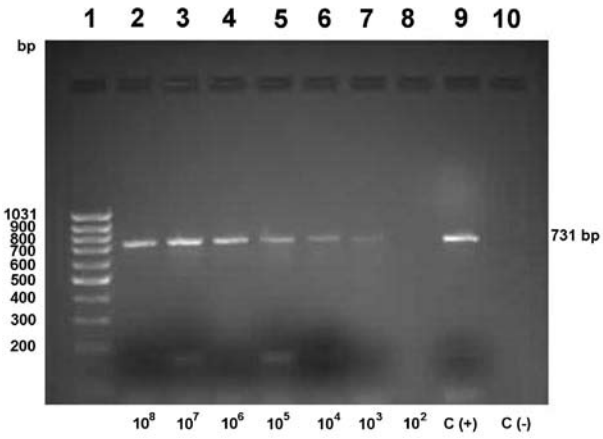


**Figure 1.** Detection limit of *B. melitensis* 16 M strain in inoculated milk by PCR assay. Line 1: Marker (GeneRuler 100bp DNA Ladder, Fermentas); Lines 2-8: containing decreasing number of *B. melitensis* 16 M per ml of milk. Line 2: $1.7x10^8$; Line 3: $1.7x10^7$; Line 4: $1.7x10^6$; Line 5: $1.7x10^5$; Line 6: $1.7x10^4$; Line 7: $1.7x10^3$; Line 8: $1.7x10^2$ cfu/ml; Line 9: control positive (based on DNA from *B. melitensis* 16 M strain); Line 10: control negative (DNases and RNases free water, AppliChem).

Límite de detección de la cepa *B. melitensis* 16 M inoculada en leche, mediante la prueba de PCR. Línea 1: Marcador (Escalera de GeneRuler 100bp DNA, Fermentas); Línea 2-8: Contenido decreciente de *B. melitensis* 16 M por ml de leche. Líneas 2: $1,7x10^8$; Línea 3: $1,7x10^7$; Línea 4: $1,7x10^6$; Línea 5: $1,7x10^5$; Línea 6: $1,7x10^4$; Línea 7: $1,7x10^3$; Línea 8: $1,7x10^2$ ufc/ml; Línea 9: Control positivo (basado en ADN de la cepa *B. melitensis* 16 M); Línea 10: Control negativo (Agua libre de Dnases y Rnases, AppliChem).

DETECTION LIMIT OF THE PCR

A positive PCR result on the ethidium bromide stained agarose gel was detected with different aliquots containing *B. melitensis* at a density of at least $1.7 \times 10^3$- $1.7 \times 10^4$ cfu/ml in milk (figure 1).

## DISCUSSION

*Brucella* are fastidious and relatively slow growing organisms (Stack *et al* 2002). There are many selective media for the primary isolation of *Brucella* from grossly contaminated clinical materials, such as milk samples (Leal-Klevezas *et al* 1995, Hamdy and Amin 2002). In this study, *Brucella* was not isolated from milk samples cultured on blood agar and blood base Nº 2. However, the microorganism was isolated in 8 out of 102 milk samples using Farrell's modified serum dextrose agar. We suggest that this selective agar is an appropriate medium for the primary isolation of *B. melitensis* in the milk samples of sheep.

In this study, 8 (7.8%) *Brucella* strains were determined as *B. melitensis* biovar 3. This is the first report regarding the typing of *Brucella* strains isolated from sheep milk in the region of Van, in East Anatolia, Turkey. This finding suggests that *B. melitensis* biovar 3 is the predominant strain isolated in the milk of sheep after abortion in East Anatolia. This finding is in accordance with the results of Erdenliğ and Şen (2000) who informed that of 78 *Brucella* strains from different regions of Turkey, 88.5% were of *B. melitensis* biovar 3. In another report from Turkey, 39 *Brucella* isolates from sheep materials in Central Anatolia in Turkey, 94.8% were also of *B. melitensis* biovar 3 (Güler *et al* 2003).

Bacteriological isolation of *Brucella* and its identification has been described as the "gold-standard", the most reliable diagnosis of brucellosis (Alton *et al* 1975). In this study, 8 milk samples from which *B. melitensis* biovar 3 was isolated were accepted as true positive. The diagnostic sensitivity of the PCR was extreme (100%) compared with MRT (75%). The diagnostic specificity was determined as 81.3% for PCR and 75% for MRT. Since so little data is available for milk-PCR and MRT, the results of sensitivity and specificity obtained from this study could not be discussed.

*B. melitensis* species-specific primers were used in this research since previous studies in the Van region (Gürtürk *et al* 1995) and in other regions of Turkey (Arda *et al* 1987, Kenar *et al* 1990, Güler *et al* 2003) indicated that only *B. melitensis* strains were isolated from aborting ewes and in none of them were isolated *B. ovis* strains.

It has been reported that the detection limit of the PCR in milk samples range from 10 bacteria/ml (Leal-Klevezas *et al* 1995), 1000 cfu/ml (Hamdy and Amin 2002), 2.8 x $10^4$ cfu/ml (Rijpens *et al* 1996) to 4.2 x $10^4$ cfu/ml (Romero and Lopez-Goñi 1999). In this research, 1.7 x $10^3$- 1.7 x $10^4$ cfu/ml of *B. melitensis* 16 M strain were detected by PCR. These results suggest that the sensitivity of PCR was affected by extraction procedures. The extraction procedure applied in this research has been shown to be successful in the detection of *B. melitensis* DNA in the milk of sheep after abortion (Leal-Klevezas *et al* 2000).

A veterinary diagnostic tool using PCR-based assays has been applied to bovine milk (Leal-Klevezas *et al* 1995, Romero and Lopez-Goñi 1999, O'Leary *et al* 2006) and caprine milk (Leal-Klevezas *et al* 1995, Leal-Klevezas *et al* 2000). However, insufficient data are available to evaluate the performance of the assays for the detection of *Brucella* DNA from ovine milk samples. In a study by Hamdy and Amin (2002), 103 milk samples were collected from 52 cows, 21 ewes, 18 goats and 12 camels. The milk samples were tested by culture, PCR and MRT. The PCR assay amplified *Brucella* DNA from 53 milk samples and the direct culture method detected *Brucella* organisms from 47 samples. 73 milk samples were evaluated as positive by MRT. In agreement with our results (102 samples, 24/8), Leal-Klevezas *et al* (2000) also detected as positive a higher number of milk samples by PCR assay when compared to bacteriological culture methods (17 samples, 11/0). This was probably because *Brucella* was present in very low numbers, which would be consistent with the small number of colony forming units detected in milk samples by culture methods (O'Leary *et al* 2006). In the present study, the agreement between PCR and MRT was 96% and when the results of PCR and MRT were compared, a higher number of samples were evaluated as positive by MRT than PCR assay (28/24) and this is partially similar to the results (103/73) of Hamdy and Amin (2002). MRT is the most widely used test for monitoring and screening brucellosis in dairy cattle (Alton *et al* 1988). In this study, a high positive result with MRT could be due to a lack of specificity of the test for the diagnosis of brucellosis in sheep milk (Alton *et al* 1988) or various milk conditions such as mastitis, colostrum and milk at the end of the lactating cycle (OIE Manual 2000). Another reason lies in the underlying principle of these assays, the MRT detects a serological response whereas both the PCR and culture detect the organism itself. Therefore, the animal must be shedding the bacteria in the milk in order to detect them by these methods. Moreover, serological tests give false-positive results due to cross-reactions with other bacteria including *Yersinia enterocolitica* O:9, *Campylobacter fetus, Vibrio cholera, Bordetella bronchiseptica* and *Salmonella* species (Alton *et al* 1988, Quinn *et al* 1994).

The PCR results achieved in this study are in agreement with the results obtained in previous studies, i.e. less variable than the results of bacteriology or serology (Leal-Klevezas *et al* 1995, Leal-Klevezas *et al* 2000, Hamdy and Amin 2002). Deficient isolation techniques or the stage of infection may explain the superiority of the PCR assay to isolation methods. Moreover, PCR assay detects both living and dead organisms, while bacteriological culture methods detect only living organisms.

The specificity of the primers used in the current study has been evaluated with a variety of microorganisms that have a close antigenic relationship with *Brucella* which causes false-positive results in serology, and the absence of amplification with DNA of these species has shown the primers to be specific for *B. melitensis* biovars 1, 2 and 3 (Bricker and Halling 1994).

The determination of *B. melitensis* from sheep milk samples is important in production of Herby Cheese (*Otlu Peynir*), one of the popular cheeses traditionally produced from raw milk of sheep, goat and cow or a mixture of these in eastern Turkey, including the Van region (Ekici *et al* 2006). Thus, Herby Cheese may be a route for transmission of brucellosis to humans. Therefore, control of the brucellosis in animals should lead to decreased incidence of the disease in humans.

In this study, *B. melitensis* DNA was detected in 24 (23.5%) out of 102 milk samples by PCR, while only 8 (7.8%) samples positive by bacteriological isolation methods. This indicated that the sensitivity of the PCR assay was higher than that of the culture method. The results achieved in this study are in agreement with the data obtained in previous works (Leal-Klevezas *et al* 1995, Romero *et al* 1995, Hamdy and Amin 2002). The described PCR has several advantages over the bacteriological culture methods, since the PCR is more rapid and sensitive than bacteriological culture methods and the amount of milk used for assay is much more smaller than that required for culture methods. In conclusion, PCR assay is a very useful tool for the rapid diagnosis of *B. melitensis* in sheep milk.

## SUMMARY

Laboratory diagnosis of brucellosis is generally performed by microbiological and serological methods. PCR assay is a specific and sensitive choice for the detection of different bacterial agents. An evaluation of this test was carried out for the detection of *Brucella melitensis* DNA in sheep milk. 102 milk samples from sheep after abortion were taken and studied using bacteriological culture, PCR and milk ring test (MRT). PCR found *B. melitensis* DNA in 24 (23.5%) out of 102 milk samples, while only 8 (7.8%) of the samples were positive to *B. melitensis* through direct culture. MRT found 28 (27.4%) positive milk samples. The detection limit for PCR in sheep milk inoculated with *B. melitensis* strain 16 M was $1.7x10^3$–$1.7x10^4$ cfu/ml. PCR and MRT coincidence was 96%. The diagnostic sensitivity and specificity were determined as 100% and 81.3% respectively for PCR assay and 75% and 75% for MRT. PCR is a useful tool for a fast diagnosis of *B. melitensis* in sheep milk.

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***Valdivia - Chile***
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