ORIGINAL ARTICLE

Comparison of culture and PCR for the detection of *Brucella melitensis* in blood and lymphoid tissues of serologically positive and negative slaughtered sheep

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Abstract

Aims: To compare the culture and PCR methods for detection of *Brucella melitensis* in blood and lymphoid tissue samples obtained from slaughtered sheep ($n = 162$) testing positive/negative in serological tests (Rose Bengal test and serum agglutination test).

Methods and Results: Of 162 sheep examined, 45 were positive and 117 negative in serological tests. A PCR assay based on a pair of *Br. melitensis*-specific primers was used to detect DNA in blood and lymphoid tissue. *Brucella melitensis* was isolated from 1·2% (2/162) and 17·2% (28/162) of the blood and lymphoid tissue samples respectively. Positive PCR products with a molecular size of 731 bp were obtained from 27·7% (45/162) of blood and 29·0% (47/162) of lymphoid tissue samples.

Conclusions: The species-specific PCR assay detected a higher number of *Br. melitensis* DNA both from serologically positive ($P < 0·01$ in blood PCR, $P < 0·001$ in tissue PCR) and serologically negative ($P < 0·001$ in both blood PCR and tissue PCR) sheep compared with classical bacteriological culture methods.

Significance and Impact of the Study: The results emphasize the importance of using more than one type of diagnostic technique for the detection of animals positive for brucellosis, especially with epidemiological purposes.

Introduction

Brucellosis in sheep and goats caused by *Brucella melitensis*, one of the most virulent species of *Brucella*, is a widespread zoonosis, especially in Mediterranean and the middle-east regions where it also constitutes a hazard for humans (Jacques *et al.* 1998). This micro-organism is the main aetiological agent of sheep brucellosis in Turkey (Arda *et al.* 1987; Leyla *et al.* 2003).

Brucellosis spreads between animals in a herd and the disease is a systemic infection that can involve many organs and tissues. Once the acute period of the disease is over, symptoms of brucellosis are mostly not pathognomonic, and the organism can be chronically located in the supramammary lymphatic nodes and mammary glands of 80% of infected animals. Thus they continue to secrete the *Brucella* organism in their body fluids (Cordes and Carter 1979; Redkar *et al.* 2001).

Nucleic acid-based amplification techniques such as PCR have been shown to be valuable methods for detection of DNA from different bacteria, providing a promising option for the diagnosis of brucellosis. PCR has been applied to a wide range of goat samples such as blood (Leal-Klevezas *et al.* 1995; Gupta *et al.* 2006), milk (Leal-Klevezas *et al.* 1995; Hamdy and Amin 2002) and lymphoid tissue (Gupta *et al.* 2006); bovine samples such as blood (O'Leary *et al.* 2006) and milk (Romero *et al.* 1995; O'Leary *et al.* 2006); and sheep samples such as stomach contents of aborted foetuses (Çetinkaya *et al.* 1999; Leyla *et al.* 2003) and milk (Hamdy and Amin 2002). However, detection of *Br. melitensis* in sheep by PCR using blood and lymphoid tissue samples has received comparatively

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little attention. This study attempts to assess the potential use of PCR for the detection of *Br. melitensis* DNA by analysing blood and lymphoid tissue samples collected from serologically [Rose Bengal test (RBT) and serum agglutination test (SAT)] positive and negative slaughtered sheep.

Materials and methods

Materials

Samples were collected from 162 sheep (all female) slaughtered in the Van Branch Slaughterhouse, Meat and Fish Institute, Eastern Turkey. Of these 162 sheep, 45 tested positive and 117 negative in serological tests (RBT and SAT) for *Brucella*. The age of the sheep (from 34 different flocks) was 4–7 years. The sheep were apparently healthy at the time of slaughter. Information was available on sporadic abortions prior to sampling. None of the animals were previously immunized against *Brucella.* Strict aseptic precautions were taken during collection of samples and different disposable gloves were used for the collection of each sample. Fifteen millilitres of whole blood (without anticoagulant) was aseptically taken from the jugular vein and immediately divided into 5 ml aliquots in three sterile tubes. Two tubes with sodium citrate (as anticoagulant) were used for (i) culture and (ii) PCR. The third blood sample (without anticoagulant) was used for (iii) serological tests. After slaughter of the animals, lymphoid tissues were sampled with a sterile scalpel from the supramammary and mammary lymph sites and immediately placed in different sterile containers. All materials were cooled on ice during transport to the laboratory. Samples for PCR were kept at −80°C before processing.

Serological tests

Antigen prepared from *Br. abortus* S99 strain for RBT and SAT was supplied by Pendik Veterinary Control and Research Institute, Istanbul, Turkey. The tests were performed as described previously (Alton *et al.* 1988). Any serum sample tested positive in both RBT and SAT was accepted as serologically positive.

Bacteriological examinations

All tissues were handled using disposable gloves. A 5 g portion of lymphoid tissue samples was placed in a stomacher™ bag with 10 ml of sterile phosphate-buffered saline (pH 7·2) and homogenized for 10–15 min (O'Leary *et al.* 2006). The homogenate was divided into two (2 ml) aliquots. One series of aliquots (iv) were used for DNA extraction and the other (v) for bacteriological cultures.

One millilitre each of lymphoid tissue homogenate (v) and blood sample (i) were immediately cultured onto duplicate blood agar plates (1·10886; Merck, Darmstadt, Germany) containing 5% (v/v) defibrinated sheep blood and three blood agar base no. 2 (CM0271; Oxoid, Hampshire, UK) supplemented with *Brucella* selective supplement (SR083A; Oxoid) and 5% (v/v) defibrinated sheep blood. Lymphoid tissue cultures were incubated at 37°C, both aerated and microaerobically (5–10% CO_2), for at least 5–7 days. Blood cultures were placed in candle jars with moist paper towel in the bottom and incubated in same conditions for 28 days. Cultures were periodically checked for growth. *Brucella* strains were identified using standard classification tests including colony morphology, Gram stain, catalase, oxidase and urease activities, H_2S production, CO_2 requirement, dye inhibition tests with thionine (40, 20 and 10 $\mu g\ ml^{-1}$) and basic fuchsine (20 and 10 $\mu g\ ml^{-1}$) and slide agglutination test with monospecific A and M sera (Alton *et al.* 1988; Quinn *et al.* 1994; Stack *et al.* 2002).

DNA extraction from blood and lymphoid tissue samples

A modification of the method reported by Miller *et al.* (1988) was used for DNA extraction from blood samples and the method described by Leal-Klevezas *et al.* (1995) was used for DNA extraction from lymphoid tissue samples.

DNA pellets (blood and lymphoid tissues) were rinsed with 1 ml of 70% ethanol, dried and the final pellets were dissolved in 50 μl of TE buffer (A0386; AppliChem, Darmstadt, Germany). DNA concentration was determined spectrophotometrically (GBC; Dadenong, Victoria, Australia) by absorbance readings at 260 and 280 nm. The samples were stored at −20°C until used as templates for amplification.

Primers, amplification conditions and agarose gel electrophoresis

A 731 bp fragment was amplified with *Br. melitensis*-specific primers as described by Bricker and Halling (1994). The sequences of the primers were 5′-AAA TCG CGT CCT TGC TGG TCT GA-3′ (forward primer, *Br. melitensis*-specific primer) and 5′-TGC CGA TCA CTT AAG GGC CTT CAT-3′ (reverse primer, IS711-specific primer).

All amplifications (blood and lymphoid tissues) were performed in a total reaction volume of 50 μl containing 5 μl of 10 × PCR buffer (4503; Fermentas, Vilnius, Lithuania), 6 μl of 25 mmol l^{-1} $MgCl_2$, 1 μl (10 mmol l^{-1})

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each of the four dNTPs (Larova, Teltow, Germany), 1 μl of *Taq* DNA polymerase (5 U μl^{-1}) (4835; Fermentas), 0·5 μl of each primer (50 pmol ml^{-1}) and 5 μl of template. One microlitre of bovine serum albumin (BSA) (10 mg ml^{-1}) (A-7638; Sigma, St Louis, MO, USA) in 0·85% NaCl was added as an enhancer to blood PCR reaction mixtures. PCR reaction mixtures without BSA served as the negative controls. PCR was performed in a DNA thermocycler (PX2 Thermal Cycler; Thermo Electronic Corp., Milford, MA, USA) and amplifications were performed using an initial denaturation temperature of 94°C for 5 min, followed by 35 cycles of denaturation, annealing and extension at 94°C, 58°C and 72°C respectively (each for 60 s). The final extension was at 72°C for 10 min. Negative controls containing all PCR reagents except template DNA were used to monitor cross contamination. The positive control used was DNA isolated from *Br. melitensis* reference strain 16M, kindly supplied by the Pendik Veterinary Control and Research Institute. To check the reliability of the results and to detect any external contamination, all samples were processed in duplicate.

The amplified products were analysed by electrophoresis on 1·5% (w/v) agarose gel at 80–90 V for 1·5–2 h and stained with ethidium bromide (0·5 μg ml^{-1}). Amplified products were visualized by a computerized image analysis system (Gl-5000; Spectronics Co., Westburg, NY, USA). PCR products with molecular size 731 bp were considered positive for *Br. melitensis*.

Statistical analysis

Forty-five sheep tested positive according to the serological tests and were accepted as true positives in the statistical analyses for the calculation of sensitivity and specificity. The chi-squared test was used to evaluate statistical significance and $P < 0{\cdot}05$ was accepted as significant (Thrusfield 1986). The level of agreement between the methods (PCR and serological tests) was also analysed (95% confidence interval) using one proportion method (http://www.minitab.com, released version 15) (accessed 2 August 2007).

Results

In this study, blood and lymphoid tissue samples were collected from serologically positive (n = 45) and negative (n = 117) slaughtered sheep. In the examination of these serum samples with RBT and SAT, seropositivity was obtained in 31·4% (51/162) and 27·7% (45/162) of samples respectively. However, *Br. melitensis* was isolated from only 1·2% (2/162) and 17·2% (28/162) of blood and lymphoid tissue samples respectively. All *Br. melitensis* strains were isolated from serologically positive sheep. PCR products with a molecular size of 731 bp were obtained from 27·7% (45/162) of blood and 29·0% (47/162) of lymphoid tissue samples. Of the 45 PCR positive blood samples, 91·1% (41/45) were obtained from serologically positive sheep and 3·4% (4/117) from serologically negative sheep. Of the 47 PCR positive lymphoid tissue samples, 100% (45/45) collected from serologically positive sheep and 1·7% (2/117) from serologically negative sheep. Typical PCR results from a set of samples are presented in Fig. 1. All culture positive blood and tissue samples were also positive by PCR. Four sheep sera were negative by the serological tests but their blood was positive by PCR, while two sheep sera were negative by the serological tests but their lymph tissues were positive by PCR. On the other hand, four sheep were positive by serological tests, but their blood samples were negative by PCR (Table 1).

Statistical analysis

When the PCR results were compared with the serological test results, the sensitivity and specificity were determined

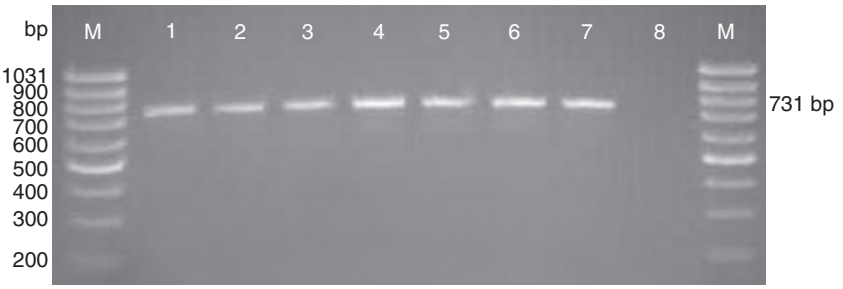


Figure 1 *Brucella melitensis* PCR products obtained from blood and lymph tissue samples separated on a 1·5% (w/v) agarose gel. M, GeneRuler™ 100 bp DNA Ladder Plus (SM0241; Fermentas); lane 1, culture positive, PCR positive blood sample; lanes 2–3, culture negative, PCR positive blood samples; lane 4, culture positive, PCR positive lymph tissue sample; lanes 5–6, culture negative, PCR positive lymph tissue samples; lane 7, control positive (based on DNA from *Br. melitensis* 16M strain); lane 8, control negative.

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Table 1 Comparative results of culture and PCR in serologically [(RBT) and (SAT)] positive and negative slaughtered sheep

	Blood				Supramammary and mammary lymph tissue			
	Culture		PCR		Culture		PCR	
Samples	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Positive* (45)	2 (4·4)	43 (95·5)	41 (91·1)	4 (8·8)	28 (62·2)	17 (37·7)	45 (100)	0 (0)
Negative† (117)	0 (0)	117 (100)	4 (3·4)	113 (96·5)	0 (0)	117 (100)	2 (1·7)	115 (98·2)
Total (162)	2 (1·2)	160 (98·7)	45 (27·7)	117 (72·2)	28 (17·2)	134 (82·7)	47 (29)	115 (70·9)

RBT, Rose Bengal test; SAT, serum agglutination test.
*RBT and SAT positive; †RBT and SAT negative.
Values are represented as *n* (%).

as 100% and 98·2%, respectively, for tissue PCR, and 91·1% and 96·5% for blood PCR. The differences between the rates of positivity in blood and tissue PCR assays were determined to be statistically significant ($P < 0·001$) within the antibody-positive animals, antibody-negative animals and all animals. Within the antibody-positive animals, there was a statistically significant difference between culture and PCR for blood samples ($P < 0·01$) and lymph tissues ($P < 0·001$). Within the antibody-negative animals, there was a statistically significant difference between culture and PCR for blood ($P < 0·001$) and lymph tissues ($P < 0·001$). Within all animals, there was a statistically significant difference between culture and PCR for blood ($P < 0·001$) and lymph tissues ($P < 0·001$). The agreement between PCR and serological tests was 1·0 for tissue PCR and SAT, 0·91 for blood PCR and SAT, 0·88 for tissue PCR and RBT and 0·80 for blood PCR and RBT.

Discussion

There are many studies that report isolation of *Brucella* from aborted animal foetuses (Çetinkaya *et al.* 1999; Leyla *et al.* 2003) and milk samples (Leal-Klevezas *et al.* 1995; Hamdy and Amin 2002). However, few studies are based on detection of *Brucella* from blood and lymphoid tissue samples. Leal-Klevezas *et al.* (1995) reported that *Brucella* was isolated from one (4%) blood sample from 24 goats, of which 14 tested positive by RBT. In another study, *Br. abortus* was isolated from only seven lymph glands whereas, blood, milk and lymphoid tissue samples collected from 21 cows, which tested positive by routine serological tests and were examined by culture (milk and lymph tissue) and conventional and real-time PCR methods (blood, milk and lymphoid tissue) (O'Leary *et al.* 2006). In our study, *Br. melitensis* was isolated from only 4·4% of blood samples and 62·2% of lymph tissue samples from sheep that tested positive in serological tests. This isolation rate from the blood is in agreement with the results reported by Leal-Klevezas *et al.* (1995). Low isolation rates could be attributed to the fact that recovery of the bacteria from blood cultures lacked sufficient sensitivity (Gupta *et al.* 2006). As concerns the lymphoid tissue, the bacteria were present at very low numbers. This is consistent with the small number of colony forming units detected in this material by classical bacteriological methods. Differences in isolation techniques, types of sample and viability of the organism in the sample may also have affected the isolation rates of the *Brucella* organisms.

To our knowledge, insufficient data are available for comparing the diagnostic performance of PCR in sheep brucellosis using blood and lymphoid tissue samples. Gupta *et al.* (2006) used PCR to analyse blood and lymphoid tissues collected from 76 goats of which 29 were positive by SAT and dot-ELISA and found 51% (39/76) and 54% (41/76) to be positive for tissue and blood PCR respectively. O'Leary *et al.* (2006) reported that blood samples collected from 21 serologically positive cows contained no *Br. abortus* in PCR assays, but they detected five positive results in tissue samples. In this research, examination of serum samples by RBT and SAT led to seropositivity being detected in 31·4% and 27·7% samples, respectively, whereas, *Br. melitensis* was isolated from 1·2% of blood and 17·2% of lymphoid tissue samples and *Br. melitensis* DNA was detected from 27·7% by blood PCR and from 29·0% by tissue PCR. These results highlight the importance of using more than one type of diagnostic technique for the detection of animals positive for brucellosis, especially with epidemiological purposes.

In this study, tissue PCR for both serologically positive and negative sheep gave a higher proportion of positive results than blood PCR. The difference between the rates of positivity in tissue PCR and blood PCR assays was determined to be statistically significant within the antibody-positive animals ($P < 0·001$), antibody-negative animals ($P < 0·001$) and all animals ($P < 0·001$). We suggest that PCR analysis of the supramammary and mammary lymph tissue is a better way to detect *Br. melitensis* DNA

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than analysis of the whole blood samples. This is important for the detection of *Br. melitensis* by PCR using lymphoid tissue samples from postmortem sheep. There may be number of reasons why tissue PCR for the detection of *Brucella* DNA is superior to blood PCR. The stage of infection may very well influence the number and location of *Brucella* organisms in white blood cells and lymphoid tissue glands. Indeed, we did not know the stage of infection of sheep in this study. However, the sheep were diagnosed as positive for *Brucella* antibodies by RBT and SAT and therefore, they evidently had an increased humoral immune response for some time. Whereas animals shed *Brucella* organisms in their blood at the early stages of the infection (Jones *et al.* 1997), materials used in this study might have been collected during later stages of the infection. The fact that the abattoir materials in the region of Van would mostly represent unproductive and undernourished sheep might have also contributed to this finding. Another plausible explanation for these differences can be that the presence of different inhibitory substances in whole blood (i.e. anticoagulants, haemoglobin and any other inhibitors) is higher than that in lymphoid tissue. In this research, positive and negative control samples were included with the test samples throughout processing by PCR. Addition of BSA to the blood PCR reaction mixtures was shown to slightly decrease the PCR inhibition in the PCR assay with samples from the serologically-positive sheep.

The diagnosis of brucellosis in small ruminants and cattle requires the use of more than one serological test. CFT, RBT and SAT are among the most useful tests for routine diagnosis (Baum *et al.* 1995) and thus RBT and SAT were used to detect *Brucella* antibodies in this study. The results showed excellent (1·0) agreement between the results of tissue PCR and SAT, but agreement between the results of blood PCR and SAT (0·91), tissue PCR and RBT (0·88) and blood PCR and RBT (0·80) was poor in serologically-positive sheep. It should be borne in mind that the serologically positive/PCR positive samples contained infectious bacteria and this has clinical/environmental significance.

An ideal diagnostic method should be specific, sensitive, fast, inexpensive and easy to perform. At present the PCR assay described fulfils all these requirements except that it is fairly expensive, but reagent cost may decrease as more PCR diagnostic assays are developed. PCR has a significant safety advantage over culture, and could provide results in <24 h. In this study, the sensitivity and specificity were determined as 100% and 98·2% respectively for tissue PCR and 91·1% and 96·5% for blood PCR. Indeed, of the 45 serologically positive sheep, 41 and 45 were positive by blood PCR and tissue PCR respectively.

In conclusion, we emphasize the importance of using more than one type of diagnostic technique for the detection of animals positive for brucellosis, especially for epidemiological purposes. The results indicated that the species-specific PCR assay can detect higher numbers of *Br. melitensis* DNA from both serologically-positive and negative sheep than classical bacteriological culture methods.

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