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Papers

Comparison of direct culture versus PCR for the detection of *Brucella* in aborted fetuses of cattle and sheep in Turkey

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The aim of this study was to detect *Brucella* in samples from aborted fetuses of sheep and cattle in Turkey using PCR and bacteriological analysis, and to determine the sensitivity and specificity of the PCR. Organ homogenates from 38 aborted fetuses of cattle and 56 aborted fetuses of sheep were tested. All organ homogenates were cultured for bacteriological analysis, and all of the homogenates and the *Brucella* isolates obtained by culture were examined with a commercial PCR kit. On bacteriological analysis, *Brucella* species were found in 30 (31.9 per cent) of the 94 organ homogenates, eight (21.1 per cent) of which were from cattle and 22 (39.3 per cent) from sheep. Using PCR, a total of 29 (30.9 per cent) homogenates were positive for *Brucella* species, eight (21.1 per cent) of which were from cattle and 21 (37.5 per cent) from sheep. Compared with the bacteriological method, the diagnostic sensitivity and specificity of the PCR kit used in this study were 83 per cent and 94 per cent, respectively.

BRUCELLOSIS is a zoonotic infection that threatens human and animal health and has an important economic impact on livestock worldwide. The prevalence of the disease in human beings is inversely correlated with the socioeconomic status of the country (Mediterranean Zoonoses Control Programme of the World Health Organization [MZCP] 1998, Pappas and others 2006). When the disease becomes endemic in a country, its government is responsible for organising a programme for eradication and control (MZCP 1998, European Food Safety Authority [EFSA] 2008).

In Turkey, the annual number of reported cases of human brucellosis infection has increased, exceeding 15,000 in 2004 (Pappas and others 2006). The disease affects cattle, sheep, goats and human beings (Erdenlig and Sen 2000, Refai 2002, Buyukcangaz and others 2009), despite enhanced application of the Turkish Brucellosis Challenge Project, which has been running for the past 20 years.

Standard isolation procedures are time consuming and require skilled personnel to carry out complex identification tests and to maintain biosafety standards during the handling of *Brucella*, a highly dangerous class III pathogen. To overcome the difficulties in the detection of *Brucella*, simple genus-specific PCR assays are potentially useful for preliminary screening. Epidemiological investigations require simpler, faster and more sensitive procedures. PCR is sensitive enough to detect low levels of bacteria both early and late in the infection cycle. It is rapid, easy to perform and inexpensive (Bricker 2004). Previous studies have demonstrated that PCR can be used to detect *Brucella* DNA in clinical samples (Leal-Klevezas and others 1995, Cetinkaya and others 1999, Romeo and Lopez Goni 1999, Cortez and others 2001, O'Leary and others 2006). Moreover, for large-scale screening of animals, PCR-based assays have been found to be useful in the diagnosis of brucellosis (Leal-Klevezas and others 1995, Romeo and Lopez Goni 1999).

In the present study, the authors attempted to identify suspected *Brucella* isolates directly by application of a *Brucella*-specific PCR kit (Bru-Com Vet; Sacace Biotechnologies) in aborted fetuses of cattle and sheep.

Materials and methods

Samples

Aborted fetus samples, including the lung, spleen, heart and liver, from 56 sheep and 38 cattle were collected from different herds in the Marmara region of Turkey during two lambing/calving seasons between 2005 and 2007. Five samples each from different *Brucella*-free and *Brucella*-positive sheep and cattle herds, as confirmed by ELISA (Svanovir C-ELISA; Svanova Biotech) and bacteriological analysis, were also used as negative and positive controls. Serum samples from animals that had aborted were not provided regularly, except for the controls. Internal organs (lung, liver, heart and spleen) from each aborted fetus were pooled together and PBS was added in 50 ml sterile vials. The samples were homogenised with an Ultra-Turrax Miccra RT-D9 (ART Prozess- & Labortechnik) (Alton and others 1975, 1988). Internal organs were not sampled independently; therefore, in total, 94 homogenates were sampled from 94 different fetuses. The homogenates were stored at −20°C before analysis. The breeds and rearing systems of the animals were not considered in the study.

Bacteriological examination

Polyclonal antisera and reference strain

Anti-*Brucella* polyclonal serum, monospecific A and M antisera and the reference *Brucella* strains, that is, *Brucella melitensis* biotype 1 (16 M-ATCC 23456), *B melitensis* biotype 2 (63/9-ATCC 23457), *B melitensis* biotype 3 (Ether-ATCC 23458), *Brucella abortus* biotype 1 (544-ATCC 23448), *B abortus* biotype 3 (Tulya-ATCC 23450) and also vaccine

Veterinary Record (2011) 168, 430 doi: 10.1136/vr.c7003

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Provenance: not commissioned; externally peer reviewed

Published Online First April 14, 2011

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strains *B melitensis* Rev 1 and *B abortus* S19, were provided by the Brucella Laboratory of Pendik Veterinary Control and Research Institute, Istanbul, Turkey.

Bacterial isolation and identification

Blood agar base No 2 (Oxoid), containing 7 per cent defibrinated sheep blood, and *Brucella* medium base (Oxoid), containing *Brucella* selective supplement (Oxoid), were used for isolation. Samples from each of the 94 aborted fetus organ homogenates were used for culture on agar plates and incubated at 37°C under aerobic conditions with 5 per cent carbon dioxide for three to eight days. Colony morphology and opacity were detected using a stereomicroscope (model SZ61; Olympus) and examined with acriflavine (Sigma-Aldrich). Oxidase tests were performed by the Kovacs method. Urease activities of the isolates were determined in Christensen's medium. Lysis by Tbilisi phage (at routine test dilution), requirement for added carbon dioxide, growth in the presence of thionin (20 µg/ml) and basic fuchsin (20 µg/ml), production of hydrogen sulphide, oxidase and urease activity and agglutination with monospecific A and M antisera were investigated for biotyping. Growth features in 2.5 µg/ml streptomycin and 5 iu/ml penicillin were examined for differentiation between field strains and the vaccine strains Rev 1 and S19, respectively. Serum dextrose agar was used as base media for dye and antibiotic susceptibility tests (Alton and others 1975, 1988, Buyukcangaz and others 2009, OIE 2009a, b).

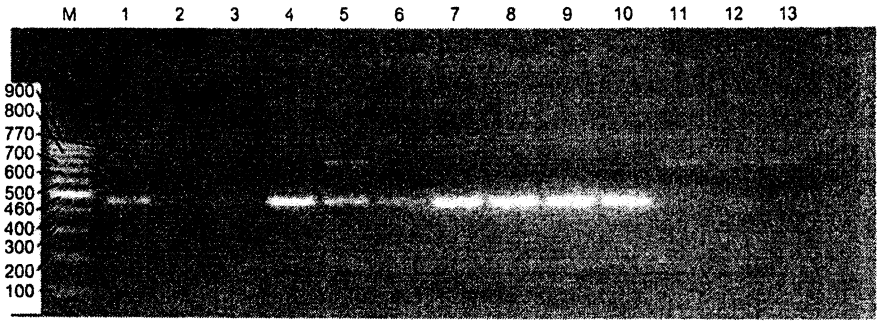


FIG 1: *Brucella* PCR products obtained from homogenates separated on a 2 per cent (w/v) agarose gel. M GeneRuler 100 bp DNA Ladder Plus (SM0241; Fermentas), Lane 1 Positive control, Lane 2 Negative control and internal control (770 bp), Lane 3 Buffer control, Lane 4 Reference strain 16M, Lanes 5 to 7 Positive homogenates, Lanes 8 to 10 *Brucella* isolates, Lanes 11 to 13 Negative homogenates

PCR

Extraction of genomic DNA from organ homogenates and *Brucella* isolates for PCR

A Bru-Com Vet (Sacace Biotechnologies) commercial kit was used for DNA extraction. Briefly, frozen homogenates were thawed at 25°C and 100 µl of each sample was added to the appropriate tube containing a lysis solution (300 µl) and an internal control (10 µl). The reagents were prepared according to the kit protocol. A volume of 100 µl of a negative control was added to a tube labelled Cneg. Samples were dissolved in lysis solution and incubated at 65°C for five minutes. After centrifugation for five minutes at 14,000*g*, the supernatants were transferred to new tubes for DNA extraction. A volume of 20 µl of the sorbent provided in the kit was added to each tube. Next, all tubes were vortexed for five to seven seconds and incubated for three minutes at 25°C. This step was repeated twice, and the supernatants were removed carefully after centrifugation at 5000*g* for 30 seconds. Two washing solutions were added to the tubes, the first at 300 µl and the second at 500 µl. The tubes were vortexed and centrifuged for 30 seconds at 8000*g* after adding each washing solution. The supernatants were discarded, and all tubes were incubated with the caps open for five minutes at 65°C. Finally, the DNA pellets were resuspended in 50 µl of DNA eluent and centrifuged. Extracted DNA from each homogenate was aliquoted and stored at -20°C until further use.

Fresh cultures made from 30 *Brucella* isolates obtained from culture of the organ homogenates were homogenised in individual tubes containing 2 ml sterile PBS. The homogenates were vortexed vigorously, and 100 µl of each sample was used to prepare DNA templates. The same procedure was used for extraction of genomic DNA from the isolates.

Primers

There was no information about the sequences of primers included in the commercial kit for reasons of confidentiality. Primers were used to amplify a 460 bp target sequence within a gene encoding *Who-A* (glycosyltransferase), which is specific to the *Brucella* genus (S. A. Algingil, personal communication). The commercial kit was not designed to distinguish between vaccine strains of *Brucella* species (Rev 1 and S19). The kit contained a 770 bp internal control that serves as an amplification control for each individually processed sample to identify possible reaction inhibition.

Amplification and detection of *Brucella* DNA by PCR

PCR was performed according to the kit protocol. Briefly, amplification mixtures were prepared by adding 10 µl of PCR-mix-2 supplied in the kit into each of the required number of ready-to-use, single-use PCR-mix-1 tubes; 10 µl of DNA sample obtained by sample preparation was added to each tube. For amplification controls, 10 µl of DNA buffer and the same volume of a positive control were used. The amplification reaction was performed on an Idaho Air Thermal Cycler (Idaho Technology) as follows: the first cycle was at 95°C for five minutes, the next 42 cycles were at 95°C (denaturation) and 65°C (annealing) for one minute each, and the last cycle was at 72°C for one minute (extension). The samples were stored at 10°C. The sizes of the amplified DNA samples were determined by electrophoresis on a 2 per cent agarose gel stained with 0.6 µl/ml ethidium bromide and compared with DNA molecular weight standards (Fermentas). DNA bands were visualised under UV light and photographed by MiniBIS-pro (DNR Bio-Imaging Systems) (Fig 1).

The sensitivity and specificity of the PCR compared with culture were calculated as described by the Clinical and Laboratory Standards Institute (2006).

Results

All colonies on the agar plates appeared to be smooth. Suspected colonies were negative on the agglutination test with acriflavine and positive for catalase, oxidase and urease. In total, 30 (31.91 per cent) of the 94 aborted fetus homogenates were bacteriologically positive.

Brucella species were isolated from eight (21.1 per cent) of 38 cattle and 22 (39.28 per cent) of 56 sheep samples). After biotyping tests and agglutination by polyclonal antiserum, all of the 22 *Brucella* isolates from sheep were identified as *B melitensis*. Of these, 15 (68.2 per cent) were identified as *B melitensis* biotype 3, six (28.5 per cent) as *B melitensis* biotype 1 and one (4.7 per cent) as *B melitensis* biotype 2. One ovine isolate identified as *B melitensis* biotype 3 was ascribed as an atypical or unusual variant because of its ability to produce hydrogen sulphide (Buyukcangaz and others 2009). Of the eight bovine isolates, seven (87.5 per cent) were identified as *B abortus* biotype 3 and one (12.5 per cent) was identified as *B melitensis* biotype 3 (Buyukcangaz and Sen 2007). In this study, none of the isolates was a vaccine strain.

Homogenates from the same aborted fetuses were also analysed using the PCR kit. In total, 29 (30.9 per cent) were positive for *Brucella* species. A total of 25 clinical samples taken from aborted cattle and sheep fetuses were positive by both bacteriology and PCR (Table 1). All isolates from cattle and sheep abortions were positive by PCR. As with bacteriology, eight of the 38 cattle homogenates were positive by PCR; however, one cattle sample was PCR-positive but culture-negative and another was culture-positive and PCR-negative. The sensitivity and specificity of the PCR kit used in this study, compared with culture results, were 83 per cent and 94 per cent, respectively.

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TABLE 1: Results of PCR and bacteriological analysis for *Brucella* species from samples of aborted fetuses

	Cattle		Sheep	
Results of culture	PCR-positive	PCR-negative	PCR-positive	PCR-negative
Culture-positive	7	1	18	4
Culture-negative	1	29	3	31
Total	8	30	21	35

Discussion

In the present study, *Brucella* infection was detected in internal organ homogenates from aborted cattle and sheep fetuses using a commercial PCR kit and bacteriological analysis. Cetinkaya and others (1999) detected *Brucella* species from the stomach contents of aborted sheep fetuses by conventional PCR. Guler and others (2003) detected *Brucella* species in the stomach contents of 126 sheep fetuses at a prevalence of 21.4 per cent by PCR and 19.2 per cent by bacteriology. Although it is not generally acceptable to take samples from the abomasums of aborted sheep fetuses because of their small size during the first and second periods of gestation, the use of PCR for rapid diagnosis of *Brucella* from the abomasal contents of aborted sheep fetuses is recommended (Guler and others 2003). Fekete and others (1992) detected *Brucella* species in 105 bovine fetal and maternal tissues using a commercial PCR kit, which, compared with bacteriological methods, had 98 per cent sensitivity and 96 per cent specificity.

The detection rates of PCR and bacteriological methods differ for several reasons. Isolation techniques, types and viability of samples, and the number and location of the organisms in the clinical sample may influence the rates of detecting *Brucella* (Cortez and others 2001, Bricker 2002, O'Leary and others 2006). Occurrences of culture-negative samples being PCR-positive could be explained by possible cross-contamination between samples or the presence of non-viable *Brucella* organisms; *Brucella* may be viable but in an unculturable state, or the bacteria in the sample may be dead (Eyigor and others 2002, Fakhr and others 2006).

PCR was repeated three times for the five culture-positive but PCR-negative samples and the same results were observed. While the bacteriological method in the present study identified a higher proportion of *Brucella*-infected animals than the PCR, other reports on the ability of PCR to detect *Brucella* species in the aborted fetuses of naturally infected animals have reported higher sensitivity and specificity for PCR than the present study (Fekete and others 1992, Cetinkaya and others 1999, Cortez and others 2001, Guler and others 2003, Ilhan and others 2007). In the present study, organs from aborted fetuses that were suspected of being heavily infected with one or more agents were used for preparing homogenates. Bacterial colonisation and large amounts of genomic ovine and bovine DNA may have had inhibitory effects on the PCR assays (Fakhr and others 2006). In addition, it may be important to extract bacterial DNA from the samples thoroughly.

Several conventional and real-time PCR assays have been established using different primers (Redkar and others 2001, O'Leary and others 2006, Bounaadja and others 2009). To date, some *Brucella* species and biovars have been defined via multiplex approaches (Ewalt and Bricker 2000, Ocampo-Sosa and others 2005, Hinic and others 2008, Ica and others 2008). Recently, a multiplex PCR that distinguishes between all *Brucella* species, including vaccine strains, was designed by García-Yoldi and others (2006). The aim of the laboratory work may determine the choice of PCR procedures: diagnostic microbiology laboratories need to be able to identify bacterial pathogens quickly for disease prevention and control reasons. Genus-specific PCR assays, as described here, could be used for the screening of livestock. The results of this study suggest that although it has a lower detection level, using a genus-specific PCR assay directly on homogenates of abortion materials might have an advantage over culture methods when used for preliminary screening, and could be valuable for the rapid detection of *Brucella* species infections.

Acknowledgements

This article is a part of the research project (V-2006/26) entitled 'Bacteriological and molecular diagnosis of *Brucella* spp in sheep and cattle abortions' that was granted by the Scientific Research Funds of Uludag University. It has been edited by American Journal Experts.

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