

DETECTION OF *Brucella melitensis* FROM FILTERED BOVINE PERIPHERAL-BLOOD BY POLYMERASE CHAIN REACTION TEST
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The term brucellosis is applied to a group of closely related infections diseases, all caused by Gram-negative bacterial pathogens in the genus *Brucella* (Moreno *et al.*, 2002). Brucellosis is an important zoonotic disease that often results in abortion and infertility in domestic animals and undulant fever, endocarditis, arthristis, and osteomyelitis in humans. The disease exists worldwide, especially in Central and South America, India, the Mediterranean basin, and the Middle East, and continues to have great health significance and economic importance in these areas (Corbel, 1997). The diagnosis of brucellosis is primarily dependent on clinical suspicion, which can be challenging since the presentation can be highly atypical (Vizcaino *et al.*, 2000). Currently, the diagnosis of this zoonosis is based on microbiological and serological laboratory tests (Nielsen 2002, Fiori *et al.*, 2000). It is well known that serological methods are not always sensitive or specific (Al-Shamahy and Wright 1998). Moreover, they have repeatedly been reported to cross-react with antigens other than those from *Brucella* spp. (Baldi *et al.*, 1996, Delpino *et al.*, 2004). Several articles describing the application of the polymerase chain reaction (PCR) technique for amplification universal genes of *Brucella* spp. have been published (Queipo-Ortuno *et al.*, 1997, Bricker 2002); and it have been reported that although some have produced false-positive results (Bogdanovich *et al.*, 2004, Morata *et al.*, 1998). This study describe a reliable, highly sensitive, and specific PCR test for *B. melitensis* detection in bovine blood. Serial dilutions of *B. melitensis* from 1×10^7 to 1×10^1 CFU per milliliter of bovine blood has been

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tested. Leucocytes filtered and cultured on Brucella agar. Finally, PCR assay for the isolates colonies using a genus-specific primer pair derived from the BCSP31K sequence of *B. melitensis*; was done.

MATERIALS AND METHODS

Bacterial strains and growth conditions: *B. melitensis* isolated from bovine milk cultures at the Microbiology/Immunology Laboratory, Atomic Energy Commission of Syria; was used in this study. *Brucella* was grown under optimal conditions in Brucella agar[®] (BD, Spark, USA) at 37°C in a water bath (Grant water bath shaker, mod, Cambridge, UK) to ensure sufficient cell density. The number of viable cells on Brucella-Agar was measured by the spread plate method, and incubation at 37°C for 24-48 h. Preparation of cell filtration and PCR: To determine the limit of detection (expressed as CFU per milliliter) of the PCR, serial bacterial dilutions of *B. melitensis* from 1×10^6 to 1×10^1 CFU per one milliliters of the dispensed blood (containing EDTA as the anticoagulant) were inoculated for 60 min at 37°C. Four hundred microliters of the sample were taken and centrifuged at 4,000 xg for 3 min. Cell pellets were resuspended in 1 ml of erythrocyte lysis solution (155 mM NH₄Cl, 10 mM NaHCO₃, 100 mM disodium EDTA [pH 7.4]), mixed, and centrifuged as described above. Treatment with erythrocyte lysis solution was repeated until the leukocyte pellets lost all reddish colour. After having been squirted (using 0.1% Triton X-100, for 10min), the leukocytes were passed onto a filter of 0.45µm. Afterwards, the filter was cultured in Petri dishes, which were incubated at 37°C for 24 and 48h. As a positive control, brucellosis culture was passed onto the filter, and cultured in a similar way as mentioned above. As a negative control we utilised a blood devoid of brucellosis, for which the complement fixation test proved negative. In addition, we have used the blood of a bovine infected with brucellosis disease, for which one complement fixation test was positive and the other was negative, in order to illustrate the increase in the test sensitivity to 20CFU/ml of blood. The isolate colony of bacteria was lysed by 3 cycles of freezing-thawing. Then, 0.6 mg of proteinase K was added and the bacteria were incubated for 1 h at 37°C. Finally boiled for 10 min. one µl of each sample was used in PCR. The BCSP31K primers 5'ACGCAGTCAGACGTTGCCTAT3' and 5'TCCAGCGCACCATCTTTCAGCCTC3', were used to amplify a 223-bp product of the BCSP31K gene. PCR was performed in a total volume of 25 µl with 1 µl of the sample, 50 pmol of each primer, 50 mM KCl, 10mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 3 mM MgCl₂, 200 µM (each) of the four nucleoside triphosphates (dNTPs), and 2.5 U of Taq polymerase (GIBCO BRL, Inc.). The reaction was performed in a DNA thermal cycler (Applied bio-system) at a denaturation temperature of 94°C for 4 min; followed by 35 cycles at 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s and one final extension at 72°C for 3 min. The PCR-amplified products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide (0.5 mg/ml), visualized under UV illumination (UVTC, Inc.) at 320 nm, and photographed.

RESULTS AND DISCUSSION

Clear visualization of PCR amplified fragments was possible in all bacterial dilution from 1×10^6 to 1×10^1 CFU, inoculated in the peripheral blood from free brucellosis bovine after electrophoresis with an agarose gel (Fig.1, lanes 5-7). The specificities and sensitivities of the amplified PCR were demonstrated from bovine with brucellosis either negative or positive complement fixation test (CF), (Fig., lanes 3 and 4); whereas the DNA amplification was negative for healthy animal (Fig.1, lane 1).

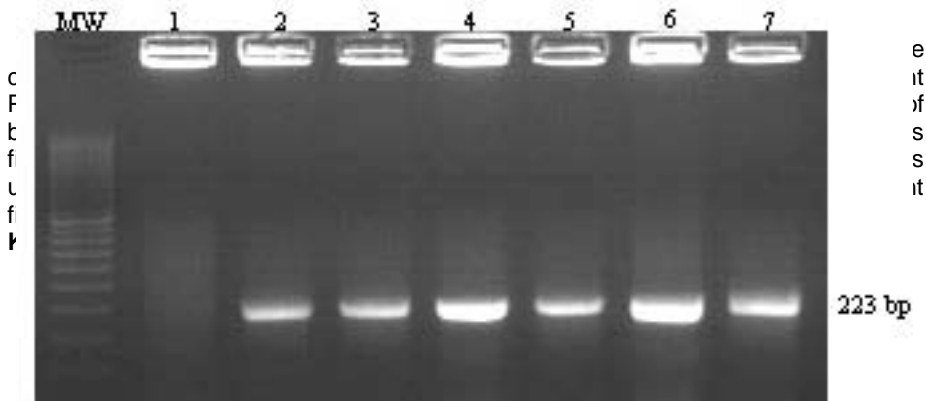


Figure 1: Electrophoresis on 1.5% agarose gel and ethidium bromide staining, showing the PCR products. Lane 1: sample from a healthy animal (as negative control), 2: positive control (*B. melitensis*), 3: bacterial DNA from bovine with brucellosis and negative CF test, 4: bacterial DNA from animal with brucellosis and positive CF test, 5-7: serial dilutions of *B. melitensis* (1×10^6 , 1×10^3 , 1×10^1 CFU respectively) inoculated in 1 ml of peripheral blood from healthy animal. Lane MW: 100-bp DNA ladder.

In the Middle East, the annual incidence of brucellosis, according to the World Health Organization reports (WHO 2004), is between 1 and 78 cases per 100,000 population; but the true figures seem to be 10 to 25 folds higher than that (Corbel, 1997). In Syria, 6860 of human brucellosis cases were registered in January 1999, and 10000 in January 2006, according to the Ministry of Health reports (personal communication).

Serology is the first diagnostic method of infections in host (Young 1995). Serological tests include: serum agglutination, a modified Coombs' technique, complement fixation, ELISAs and Western blotting (Orduna *et al.*, 2000, Anonymous 1997). Serological diagnosis is complicated by previous exposures and other factors. Chronic brucellosis can be extremely difficult to diagnose, if the serologic results are equivocal and the organism cannot be cultured (Zerva *et al.*, 2001). Although the most specific diagnostic test is the isolation of causative organism, this test need a very long time to perform because: prolonged incubation periods and specific growth media are

necessary, slow growing bacteria (up to four weeks); in addition, when other tests are positive, bacteria cultures have not be always positive (positive isolation rates are between 20-50% only) (Yagupsky, 1999). Thus, serological methods are recommended as a mean to obtain fast indirect proof of the diagnosis. Each test has its own disadvantages, and the presence of antibodies doesn't always refers to the presence of an active case of brucellosis. (Romero *et al.*, 1995, Clavijo *et al.*, 2003). Several factors were reported to inhibit PCR in a blood specimen such as high concentrations of leukocytes DNA and heme compounds (Morata *et al.*, 1998). Moreover, Navarro *et al.* (2002) reported that the presence of human DNA caused a dramatic decrease in PCR sensitivity when using primers that amplified the 16S rRNA and 31 kDA antigen of *B. abortus*. This could be as a result of competitive non-specific hybridization of the large amount of host DNA with these primers. The ideal diagnostic test for brucellosis should be easy, simple, and rapid test that will detect infected human as early as possible during the course of the disease. Thus, this study performed to develop a rapid, direct and sensitive PCR test of bovine brucellosis.

From DNA sequences obtained from the GenBank database, the conserved gene (*BCSP31K*) reported for *B. melitensis* was chosen. The sensitivity of this test was studied by using serial dilutions of *B. melitensis* in uncontaminated blood from which bacterial colony was later isolated by the method described above. Our PCR assay was highly sensitive; because it showed clear amplifications with 10^1 CFU/ml (Fig. 1, lane 7).

PCR assay for naturally and artificially infected blood yielded the same amplified fragments of 223 bp as the ones from *Brucella* colonies isolated from the corresponding infected samples (Fig. 1, lanes 3 and 7). Whereas, for the blood of healthy animal, PCR results was negative (Fig. 1, lane 1). These PCR procedures were useful for detecting the presence of the pathogen in *Brucella*-infected animals which was confirmed by CF test; also where CF test was negative (Fig.1, lanes 3 and 4). These results indicate the specificity and sensitivity of our PCR protocol for bovine *Brucella* infections. Lack of amplification when the blood incubated with *Yersinia enterocolitica* O:9 or *E. coli* O:157 supports our findings (data not shown).

In conclusion, on the basis of its sensitivity and specificity, this PCR method based on the filtered bovine blood experimentally infected by *B. melitensis*, could provide a useful diagnostic tool for brucellosis; since we could detect fewer than 20 CFU in 1 ml of blood.

This report is the first (as our knowledge) which studying the PCR test sensitivity of bacteria diluted in blood.

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الكشف عن بكتريا البروسيلة الضائنية *Brucella melitensis* من الدم
الحيواني المرشح بواسطة اختيار سلسلة تفاعلات البوليميز
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أمكن تركيز وعزل بكتريا البروسيلة الضائنية *Brucella melitensis* الملوثة لدماء
الحيوانات المصابة وذلك بعملية الترشيح الغشائي، كما أمكن فصل الحمض النووي (دنا) DNA
بواسطة اختيار PCR والكشف عنه وذلك بتسخين الخلايا لدرجة 95م لمدة 10 دقائق .
الكشف عن نسبة التلوث ببكتريا البروسيلة في العينات الملوثة للحيوان المصاب أقل من 20
خلية صلبة/مل من الدم، وذلك بعدعملية الترشيح والزرراعة عن تلك المزروعة من مستعمرات
معزولة.
طريقة PCR أيضا تمكننا من التشخيص للحيوانات المصابة بالبروسيلة بدون اثار جانبية
ضارة.

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