ACTA VET. BRNO 2010, 79: 277-280; doi:10.2754/avb201079020277

Detection of *Brucella abortus* in Bovine Milk by Polymerase Chain Reaction

Ayman Al-Mariri, Nermeen Haj-Mahmoud

Department of Molecular Biology and Biotechnology, Atomic Energy Commission, Damascus, Syria

Received October 25, 2008
Accepted December 3, 2009

Abstract

This study compares milk ring test and three different polymerase chain reaction techniques (direct DNA extraction by column purification system, alkaline DNA extraction, and filtrated milk), in order to identify *Brucella abortus* infection in bovine milk. Milk ring test sensitivity and specificity were 72% and 80%, respectively. While specificity of the three polymerase chain reaction techniques was 100%; sensitivity was 92%, 88% and 100%, respectively, for the three polymerase chain reaction procedures. We conclude that the filtered animal's milk polymerase chain reaction is the best procedure to make the diagnosis of *B. abortus* infections.

Diagnosis, milk ring test, sensitivity, specificity

Brucellosis is still one of the most common bacterial zoonoses in the Mediterranean region. The complexity of the epidemiology of brucellosis and the serious difficulties for effective control measures arise from the involvement of the main producing domestic animals (cattle, sheep, goats, camel) and humans in the infection (Boschiroli et al. 2001). This disease is caused by several species of the genus *Brucella*, a homogeneous group of small, non-motile, Gram-negative coccobacilli, and facultatively intracellular bacteria, belonging to the α-2 subdivision of the Proteobacteria (Moreno et al. 2002). The predominant symptom of an acute *Brucella abortus* infection is reproductive failure with abortion and birth of weak offspring (Fiori et al. 2000). Transmission to humans occurs by ingestion of milk products or by direct contact with tissues and fluids of infected animals (Zvizdic et al. 2006). Milk ring test (MRT) and blood serological tests are mainly used for diagnosis of the disease. Tests for detection of *B. abortus* antibodies in milk are considered the principal methods for detecting infected herds and for diagnosing brucellosis in an individual animal (Noriello 2004). Several articles describing the application of the polymerase chain reaction (PCR) technique for amplification of universal genes of *Brucella* spp. have been published. This technique could be a potentially useful method for the diagnosis of brucellosis since it could detect the bacteria in paucibacillary samples and even in samples highly contaminated with other microorganisms. In addition, PCR technique could detect more infected animals compared to serological methods (Romero and Lopez-Goni 1999; Leal-Klevezas et al. 2000; Cortez et al. 2001). It has been reported that the only unequivocal method for the diagnosis of brucellosis in host is based on the isolation of *B. abortus* bacteria (European Commission 2002; Leyla et al. 2003) at selective medium such as Farrell's selective medium (Farrell 1974). In this study, we have tried to develop a reliable molecular procedure that could increase the sensitivity and specificity of *B. abortus* detection in bovine milk.

Materials and Methods

Milk samples

Samples of milk were collected from infected cows. Fifty positive milk samples, proved by cell culture, were received from two Syrian towns (35 from Hama and 15 from Homs). Each milk sample was centrifuged at 2000 × *g* for 15 min and the cream and deposit were spread on solid selective medium (OIE Manual 2004). Brucella agar® (BD, Spark, USA) plates with 5% sterile horse serum, polymyxin B (5 U/ml), bacitracin (25 U/ml) and

Address for correspondence:
Dr. Ayman Al-Mariri
Atomic Energy Commission Of Syria (AECS)
P.O. Box 6091
Syrian Arab Republic Damascus

Fax: 00963 – 11- 6112289
Phone: 00963 -11- 2132580
E-mail: scientific@aec.org.sy
http://www.vfu.cz/acta-vet/actavet.htm

cycloheximide (100 µg/ml) were used for the isolation, identification and typing of *Brucella* spp. Plates were placed in an incubator for 48 h at 37 °C with 10% CO_2 tension adjusted automatically. Typing of *Brucella* isolates was made according to the CO_2 requirement, H_2S production, growth in the presence of dyes (thionine and basic fuchsin), and reaction with monospecific anti-A and anti-M sera (Alton et al. 1988).

Four millilitres of each remaining positive sample were combined for analysis by Milk ring test (MRT) and by three different polymerase chain reaction (PCR) protocols (direct DNA extraction by column purification system, alkaline DNA extraction and filtered milk). A total of 25 negative raw milk samples from healthy cows in addition to a pooled positive control were used to determine specificity.

Milk ring test (MRT)

This test was performed by adding 30 µl of antigen (Institute Proquier, Montpellier, France) to 1 ml of whole milk that had been stored for at least 24 h at 4 °C. The height of the milk column in the tube was at least 25 mm. A positive reaction was indicated by formation of a blue ring above a white milk column or at the interface of milk and cream. The test was considered to be negative if the color of the underlying milk exceeded that of the cream layer.

Milk filtration

Milk sample was passed onto a 0.45 µm filter. Afterwards, the filter was cultured in Petri dishes (Brucella® Agar with 20 mg/l vancomycine), which were incubated at 37 °C for 48 h. As positive control, brucellosis culture was passed onto the filter, and cultured in a similar way as mentioned above. As negative control, we utilized milk devoid of brucellosis, for which the milk ring test and cell culture proved negative. The isolated 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, the isolates were boiled for 10 min.

DNA extraction (from milk)

DNA was extracted by using a 1-ml aliquot of milk which was centrifuged at 6,000 × *g* for 10 min. The clear whey portion was suctioned out with a transfer pipette and discarded. The remaining milk solids and butterfat were used for DNA extraction using two different processing:

1. Direct DNA extraction by column purification system: Preheated sterile, double-distilled, deionized water was added till the sample volume reached 200 µl, and the mixture was vigorously vortexed to release the pellet from the bottom of the tube. A total of 25 µl of proteinase K (20 mg/ml stock) was added, and the mixture was vortexed to mix. Subsequently, 200 µl of preheated lysis buffer was added to each tube, and the contents were vortexed again until the mixture was homogeneous. The mixture was then incubated at 70 °C for 30 min. A second aliquot of proteinase K was added, and the mixture was incubated at 70 °C for additional 30 min. After incubation, 200 µl of ethanol were added, the mixture was vortexed, and the samples were processed using Wizard® SV minicolumns (Wizard® SV Genomic DNA purification System, Promega, Madison, USA) as described in the product insert. DNA was eluted in 50 µl of sterile, double-distilled, deionized water in all cases except where specified.

2. Alkaline DNA extraction: The alkaline extraction procedure was a modification of the method proposed by Daly et al. (2002). The milk pellet was resuspended in 1 ml alkaline extraction solution: (0.5 mol/l of sodium hydroxide and 0.05 mol/l of sodium citrate). This mixture was shaken for 10 min and centrifuged at 13,000 × *g* for 5 min. The supernatant fluid was removed and the pellet resuspended in 500 µl (0.5 mol/l) Tris-HCl pH 8.0, followed by centrifugation at 13000 × *g* for 5 min. This step was repeated again. The final pellet was resuspended in 100 µl (10 mmol/l) Tris-HCl, 1 mmol/l EDTA pH 8.0, then it placed in a heating block for 1 h at 100 °C. The sample was then freeze-thawed twice, centrifuged at 13 000 × *g* for 15 min and the supernatant fluid removed for PCR analysis.

PCR technique

One µl of each sample was used in PCR. The BCSP31K primers

5'-ACGCAGTCAGACGTTGCCTAT-3' and

5'-TCCAGCGCACCATCTTTCAGCCTC-3', were used to amplify a 223-bp product of the *bcsp*31K 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, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 3 mM $MgCl_2$, 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 (Applpied 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 amplification 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

Our study reveals that the *Brucella* isolates from Syrian cows belonged to *B. abortus* biovar 9. This biovar has already been reported in the Near East (Darwesh and Benkirane 2001; FAO 1998).

PCR technique could identify 46/50 samples when direct DNA extraction by column purification system was used, 44/50 cases when alkaline DNA extraction was used, and 50/50 cases when filtered milk procedure was used, with a sensitivity of 92%, 88% and 100%, respectively (Table 1). MRT could identify 36/50 cases, with a sensitivity of 72% only. PCR false-negative cases were 0/50, 4/50, 6/50 and 14/50 when filtrated milk procedure, direct DNA extraction by column purification system, alkaline DNA extraction and MRT was used, respectively.

Table 1. PCR and MRT results in milk samples taken from non-infected[1] and infected[2] animals

Tests	Non-infected	Infected	Specificity%	Sensitivity%
PCR (direct DNA extraction by column purification system)	0/25	46/50	100%	92%
PCR (alkaline DNA extraction)	0/25	44/50	100%	88%
PCR (filtrated milk)	0/25	50/50	100%	100%
MRT	5/25	36/50	80%	72%

[1] Culture was negative on *Brucella* solid selective medium
[2] Culture was positive on *Brucella* solid selective medium

In Fig. 1 (Plate XIII) the amplification of 223 bp sequence of the gene encoding the BCSP-31 antigen (lanes 4-6) as shown by the three PCR assays, ensure the presence of *B. abortus* in the examined samples. This figure reveals, in addition, the brucellosis culture that passed onto the filter (lane 3); negative control sample which represent the DNA of *Yersinia enterocolitica* O:9 (lane 1); and positive control sample from the DNA of *B. abortus* 544 (lane 2). No template was added in lane 7.

All three PCR techniques results were clearly negative for all milk samples experimentally infected with *Yersinia entrocolitica* O:9 (data not shown).

Currently**,** the diagnosis of brucellosis in bovine milk sample is based almost entirely on milk ring test, which indirectly detects *Brucella* spp. in the host (Godfroid et al. 2002). Our results reveal that the sensitivity and specificity of this test was 72% and 80%, respectively (Table 1). However, this test is limited by the milk quality and the results may be false-negative when the milk sample has low concentrations of IgM and IgA antibodies, or lacking of the fat-clustering factors (O'Leary et al. 2006). In contrast, the results may be false-positive when the milk contains colostrums and in cows vaccinated with *B. abortus* S19 or in those with mastitis.

There is increased interest to develop rapid and accurate (sensitive and specific) methods to detect *Brucella* spp. in bovine samples; and PCR procedure has been considered more sensitive and specific than serological methods (Bricker 2002; Gupta et al. 2006). Hamdy and Amin (2002) reported that PCR was a very useful procedure in the detection of the presence of wide infection in bovine milk samples. Moreover, Gupta et al. (2006) revealed that the sensitivity and specificity of PCR in detecting the presence of *B. melitensis* in goat milk were 90% and 100%, respectively; and our results showed almost the same sensitivity and specificity, especially when the bacterial DNA was isolated by column purification system, 92% and 100%, respectively (Table 1).

The extraction method of the bacterial DNA, the capacity to isolate the target bacteria, the lack of *B. abortus* colonies in some milk samples, as well as the influence of matrix components of milk could affect the sensibility and the reliability of PCR procedures (bacterial DNA isolated by column purification system, and alkaline DNA extraction). These problems were overcome in this study by passing the contaminated milk onto a 0.45 µm filter, where PCR sensibility and specificity were 100%. These results were similar to the results of bacteriological detection methods which have been proven to be the most

reliable and the only unequivocal animal brucellosis diagnostic methods (OIE 2004; Garin-Bastuji and Blasco 2004). In addition, this PCR procedure was more sensitive than direct PCR procedures (bacterial DNA isolated by column purification system, and alkaline DNA extraction). In conclusion, it is highly recommended to use this PCR procedure to identify *Brucella* in all types of milk samples. Furthermore, we advise to use this procedure as a regular screening test in farms animals.

Acknowledgements

The authors thank the Director General of AECS, and the head of the Molecular Biology and Biotechnology Department for their support. The authors also thank Dr. M. Safi for his critical reading of this manuscript.

References

Alton GG, Jones LM, Angus RD, Verger JM 1988: Techniques for the Brucellosis Laboratory. INRA. Paris.

Hamdy ME, Amin AS 2002: Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR.Vet J **163**: 299-305

Boschiroli ML, Fouonbne V, O'Callaghan D 2001: Brucellosis: a worldwide zoonosis. Curr Opin Microbiol **4**: 58-64

Bricker BJ 2002: PCR as a diagnostic tool for brucellosis. Vet Microbiol **90**: 435-446

Cortez A, Scarcelli E, Soares RM, Heinemann MB, Sakamoto SM, Genovez ME, Ferreira F, Richtzenhain LJ 2001: Detection of *Brucella* DNA from aborted bovine foetuses by polymerase chain reaction. Aust Vet J **79**: 500-501

Daly P, Collier T, Doyls S 2002: PCR-ELISA detection of *Escherichia coli* in milk. Lett Appl Microb **34**: 222-226

Darwesh M, Benkirane A 2001: Field investigations of brucellosis in cattle and small ruminants in Syria, 1990-1996. Rev Sci Tech Off Int Epiz **20**: 769-775

European Commission 2002: Regulation No. 535/2002 of 21 March 2002 amending Annex C to Council Directive 64/432/EEC and amending decision 2000/330/EC. Off J Eur Commun L 80: 22-28

FAO 1998: A Perspective of Brucellosis surveillance in north Africa and the Middle East: Studies in Algeria - Iran - Kuwait - Morocco - Oman - Sudan and Syria. January 1998; GCP/REM/059/IFA. FAO Consultancy mission report.

Farrell ID 1974: The development of a new selective medium for the isolation of *Brucella abortus* from contaminated sources. Res Vet Sci **16**: 280-286

Fiori PL, Mastrandrea S, Rappelli P, Cappuccinelli P 2000: *Brucella abortus* infection acquired in microbiology laboratories. J Clin Microbiol **38**: 2005-2006

Garin-Bastuji B, Blasco JM 2004: Caprine and ovine brucellosis (excluding *B. ovis*). In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, fifth ed. OIE pp. 598-606

Godfroid J, Saegerman C, Wellemans V, Walravens K, Letesson J-J, Tibor A, McMillan A, Spencer S, Sanna M, Bakker D, Pouillot R, Garin-Bastuji B 2002: How to substantiate eradication of bovine brucellosis when aspecific serological reactions occur in the course of brucellosis testing. Vet Microbiol **90**: 461-477

Gupta VK, Deepak K, Vermaa PK, Routa SV, Singha and Vihana VS 2006: Polymerase chain reaction (PCR) for detection of *Brucella melitensis* in goat milk. Small Rum Res **65**: 79-84

Leal-Klevezas DS, Martinez-Vazquez IO, Garcia-Cantu J, Lopez-Merino A, Martinez-Soriano JP 2000: Use of polymerase chain reaction to detect *Brucella abortus* biovar 1 in infected goats. Vet Microbiol **75**: 91-97

Leyla G, Kadri G, U¨ Mran O 2003: Comparison of polymerase chain reaction and bacteriological culture for the diagnosis of sheep brucellosis using aborted fetus samples. Vet Microbiol **93**: 53-61

Moreno E, Cloeckret A, Moriyon I 2002: *Brucella* evolution and taxonomy. Vet Microbiol **90**: 209-227

Noriello S 2004: Laboratory-acquired brucellosis. Emerg Infect Dis **10**: 1848-1850

O'Leary S, Sheahan M, Sweeney T 2006: *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Res Vet Sci **81**: 170-176

OIE Manual 2004: Bovine brucellosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE, World Organisation for Animal Health, Part 2 (Chapter 2.3.1).

Romero C, Lopez-Goni I 1999: Improved method for purification of bacterial DNA from bovine milk for detection of *Brucella* spp. by PCR. Appl Environ Microbiol **65**: 3735-3737

Zvizdic S, Cengic D, Bratic M, Mehantic S, Pinjo F, Hamzic S 2006: *Brucella melitensis:* review of the human infection case. Bosn J Basic Med Sci **6**: 15-18.

WHO 2004: Brucellosis. World Health Organization Essential Medicines Library EMLib (accessed 03/12/2004)

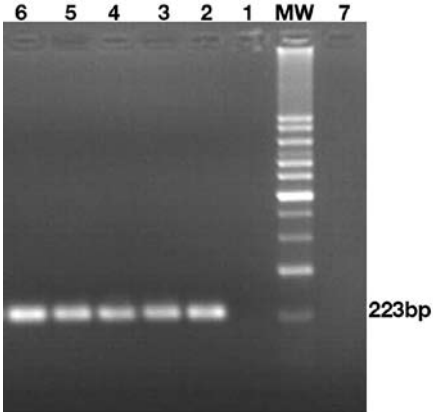

Fig. 1. Electrophoresis on 1.5% agarose gel and ethidium bromide staining, showing the results of PCR procedures. As negative control, lane 1 for DNA isolated from *Yersiunia enterocolitica* O:9, and lane 7: PCR sample without template. As positive control, lane 2: DNA of *B. abortus* 544, and lane 3: DNA was isolated from *B. abortus* 544 and passed onto 0.45 µm filter. Bacterial DNA isolated from contaminated milk samples using three protocols direct DNA extraction by column purification system, alkaline DNA extraction or filtrated milk onto 0.45 µm filter (lanes 4-6, respectively) were used as the template in PCR reactions. Lane MW: 100-bp DNA ladder.