APPLICATION OF REAL-TIME PCR FOR IDENTIFICATION AND DIFFERENTIATION OF *BRUCELLA ABORTUS* AND *BRUCELLA MELITENSIS* IN CATTLE

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Summary

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Real-time PCR is a sensitive and specific method for detection and differentiation of *Brucella* spp. This study was performed to determine the prevalence of *Brucella* spp. and to differentiate *Brucella melitensis* and *Brucella abortus* in cattle population in southwest Iran. A TaqMan analysis and single-step PCR was performed in total of 425 bovine blood samples. The results showed 127 (29.88%) positive samples for *Brucella* spp. By real-time PCR 9, 69, and 5 of these specimens, were positive for *B. melitensis*, *B. abortus*, and both bacteria respectively. Results of present study indicated a high presence of this pathogen in the region. Real-time PCR is technically more simple, accurate, and rapid than current standard methods for identification and differentiation of *Brucella* species. To our knowledge, this study is the first prevalence report of identification and differentiation of *B. abortus* and *B. melitensis* by real-time PCR technique in Iran.

Key words: *B. abortus*, *B. melitensis,* cattle, real-time PCR

INTRODUCTION

Brucellosis is believed to be an ancient disease that was described more than 2000 years ago by the Romans. Bruce was the first to isolate *Brucella melitensis* in 1887. This worldwide zoonosis is caused by infection with the bacterial genus *Brucella*, which mainly infect cows, goats, sheep, camels, pigs, swine and dogs. Though it has been eradicated in many developed countries in Europe, Australia, Canada, Japan and New Zealand (Gul & Khan, 2007), it is still highly prevalent among humans in Saudi Arabia, Iran, Palestinian Authority, Syria, Jordan and Oman (Refai, 2002). The causing agents are Gram-negative, facultative intracellular coccobacilli or short rods from the family Brucellaceae that localize in the reproductive organs of host animals, causing abortions, foetal death, genital infections (Probert *et al.*, 2004) and sterility. The transmission of the disease is by direct or indirect contact with infective excretors. They are shed in large numbers in the animal urine, milk, placental and other fluids (Otlu *et al.*, 2006; Zvizdic *et al.*, 2006).

Most species of *Brucella* can infect animals other than their preferred hosts, when they come in close contact. *B. melitensis*, *B. suis*, *B. abortus*, and *B. canis* are human pathogens. There are few different

species of *Brucella*, each with slightly different host specificity: *B. melitensis* which infects goats and sheep, *B. abortus* which infects cattle, *B. suis* infects pigs, *B. ovis* infects sheep (Romero *et al.*, 1995).

B. melitensis can affect most domestic animals, but goats and sheep are especially susceptible. *B. melitensis* and *B. abortus* are the most important species in terms of prevalence and morbidity in humans and domestic animals. Moreover, bovine brucellosis, caused by *Brucella abortus*, is a significant problem for both public and animal health in Iran (Moradi *et al.*, 2006).

To detect the presence of *Brucella* organisms in tissues, cultural, serological and PCR methods were used. Culture methods are well established for brucellosis but highly dangerous to laboratory workers, difficult and lengthy processes that requires experienced technicians. Culture often takes weeks to achieve observable growth depending on the sample type, freedom from overgrowth by other fungal and bacterial contaminants and the specific serovar causing infection (Kazemi *et al.*, 2008). The serological methods are usually employed for diagnostics of *Brucella* in blood specimens. The serological response, however, can be unspecific due to cross-reaction or subsensitive reactions in samples from areas with a low or subclinical prevalence of brucellosis (Bogdanovich *et al.*, 2004). These techniques could be potentially useful for the diagnosis of brucellosis since they could detect the bacteria in paucibacillary samples and even in samples highly contaminated with other microorganisms.

Therefore, molecular diagnostic techniques such as real-time PCR that are simpler, faster, less hazardous and usually more sensitive, have been developed for *Brucella* detection (Bricker, 2002).

Although several *Brucella* genus specific assays were also described, none of them differentiates between different species (Romero *et al.*, 1995). The routine identification and differentiation of *Brucella* species is based on phenotypic traits, but it is associated with a high risk of laboratory-acquired infections and is very time-consuming (Navarro *et al.*, 2004; Carver *et al.*, 2005). Many molecular methods – PCR restriction fragment length polymorphism, random amplified polymorphic DNA analysis – are available for differentiation of six *Brucella* species on the basis of size of PCR product (Bricker & Halling, 1994; Bricker, 1999; Tcherneva *et al.*, 2000). At present, there are several conventional and real-time PCR assays for differentiation between *Brucella* species (Foster *et al.*, 2008).

The purpose of this study was to investigate the presence and prevalence of *Brucella* spp. in southwest Iran and to differentiate *B. abortus* and *B. melitensis* using real-time PCR technique for the first time.

MATERIALS AND METHODS

Samples collection and DNA extraction

A total of 452 cattle were selected in 2 provinces (248 in Isfahan and 204 in Chaharmahal Va Bakhtiari) located in southwest Iran. The blood samples (approximately 8 to 10 mL) were obtained from the tail vein of cows and stored in 10% 0.5 M EDTA-coated vacutainer tubes (BD Vacutainer Systems, Plymouth, UK). DNA was extracted using a genomic DNA purification kit (Invitrogen, Paisley, U.K.) according to the manufacturer's recommendation. The total DNA was measured at 260 nm wavelength according to the

method described by Sambrook & Russell (2001).

Conventional PCR assay

Oligonucleotide primers used to screen the *Brucella* spp. were designed on the basis of DNA sequence of the gene coding the outer membrane protein (*omp*-2) reported for *Brucella* in GenBank database (Leal-Klevezas *et al.*, 1995). The forward primer sequence was 5'-GCGCTCAGG-CTGCCGACGCAA-3', and the reverse primer sequence was 5'-ACCAGCCAT-TGCGGTCGGTA-3'. All oligonucleotide primers were obtained from a commercial source (Fermentas, Germany).

The target sequence was amplified in a 25 µL reaction volume containing 2 µL of DNA sample, 0.5 mM $MgCl_2$, 0.2 mM dNTP, 0.8 µM of each primer and 0.5 U/reaction of *Taq* DNA polymerase. Reactions were initiated at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min and a final elongation step at 72 °C for 5 min, with a final hold at 4 °C in a DNA thermal cycler (Mastercycler Gradient, Eppendrof, Germany). A negative control (sterile water), and a positive control DNA from *B. abortus* strain S19 (spontaneously atte-nuated strain used for vaccination of cat-tle), were included in each amplification run.

Amplified samples were analyzed by electrophoresis (120 V/208 mA) in 1.5% agarose gel. The gel was stained with 0.1% ethidium bromide (0.4 µg/mL) and viewed on UVIdoc gel documentation systems (UK).

Real-time PCR assay

The real-time PCR for species differentia-tion was based on unique genetic loci of *B. melitensis* and *B. abortus*. The regions were chosen for the construction of pri-mers and TaqMan® probes for species differentiation: *BMEII0466* gene for *B. melitensis* and *BruAb2_0168* gene for *B. abortus* (Table 1).

A typical 25 µL reaction contained: 12.5 µL TaqMan® Universal PCR Master Mix (Roche Applied Science, Indianapo-lis, IN, USA), a 300 nM concentration of each forward and reverse primer (BioNeer Corporation, South Korea), a 200 nM concentration of the probes labeled with FAM and Cy5 (BioNeer Corporation, South Korea), and 2.5 ng of sample DNA. TaqMan real-time PCR reactions were carried out using a RotorGene 6000 in-strument (Corbett Research). The reaction mixture was initially incubated for 10 min at 95 °C. Amplification was performed for 45 denaturation cycles at 95 °C for 20 s, annealing and extension at 62 °C for 1 min.

Table 1. Real-time PCR primers and TaqMan® probes

	Target sequence	
	BMEII0466	*BruAb2_0168*
Primers (5′→3′)	TCGCATCGGCAGTTTCAA-CCAGCTTTTGGCCTTTTCC	GCACACTCACCTTCCACAACAA-CCCCGTTCTGCACCAGACT
Probe (5′Fluorophore →3′Quencher)	Cy5-CCTCGGCATGGCCCG-CAA-BHQ-2	FAM-TGGAACGACCTTTGCAGG-CGAGATC-BHQ-1
Fragment size	112 bp	222 bp

RESULTS

In the current study, 452 blood samples of cattle from two provinces of Iran were tested for *Brucella* spp. using a conventional PCR assay. The positive samples were analyzed by real-time PCR for identification and differentiation of *B. melitensis* and *B. abortus*.

Agarose gel electrophoresis of the amplification products showed the presence of 113-bp DNA fragment for *Brucella* spp. The presence of *Brucella* DNA was detected by single PCR in 127 of 452 animals (29.88%).

The results of the prevalence of *Brucella* spp in cattle from each province are shown in Table 2. After real-time PCR, the *BMEII0466* and *BruAb2-0168* genes were distinguished in 9 (*B. melitensis*), 69 (*B. abortus*), and 5 (both bacteria) of the 127 positive specimens. On the other hand, using *BMEII0466* and *BruAb2-0168* gene specific primers, none of these two species (*B. melitensis* and *B. abortus*) were found in 25 samples (5.53%) (Table 2).

DISCUSSION

Rapid, definitive and accurate diagnosis of brucellosis is very important for a positive outcome of eradication programmes (Surucuoglu *et al.*, 2009). Real-time PCR assays are now easy to perform, highly sensitive, and provide more specificity for detection of microorganisms. PCR and real-time PCR are promising alternatives for the problematic culturing and identification of *Brucella* spp. by conventional techniques.

The results showed that real-time PCR is a sensitive and specific method for detection and differentiation between *B. abortus* and *B. melitensis.* The advantages of this technique are that it can be performed very quickly, does not require electrophoretic analysis, and it is not contaminated as conventional PCR.

In Iran, the prevalence of brucellosis in animals attained 44% in 1956 and dropped to 5% in 1958. In 1980 and 1991, the prevalence rates were 6.4% and 10.18% (Refai, 2002). The prevalence of human brucellosis in different parts of Iran varied from 1.5 up to 107.5 per 100,000 in 2003. The highest levels of infection appeared in Hamedan with 107.5, Kurdistan with 83.5, Azarbaijan Gharbi with 71.4 and Zanjan with 67.1 per 100,000 people (Moradi *et al.*, 2006). The prevalence rate of brucellosis among horses in northeast Iran between May 2008 and April 2009 was 2.5% (Tahamtan

Table 2. Distribution of *B. melitensis*, *B. abortus* and *Brucella* spp. in southwest Iran

Province	Number of samples	Conventional PCR assay, number (%)	Real-time PCR assay, number (%)			
			B. melitensis	*B. abortus*	Both bacteria	Unknown
Isfahan	248	76 (30.64)	6 (2.41)	41 (16.53)	3 (1.20)	14 (5.64)
Chaharmahal Va Bakhtiari	204	54 (26.47)	3 (1.47)	28 (13.72)	2 (0.98)	11 (5.39)
Total	452	127 (29.88)	9 (1.99)	69 (15.26)	5 (1.10)	25 (5.53)

et al., 2010). In the same region, Bokaie *et al.* (2009) reported a brucellosis prevalence of 3.4% in sheep and goats and 0.56% in cattle.

In 2007, the prevalence of *B. melitensis* in aborted sheep in Turkey was 29.76% (Sahin *et al.*, 2008) and among cattle in Punjab (India) – 18.26% (Aulakh *et al.*, 2008). The research results of Kaoud *et al.* in 2010 pointed out that brucellosis was found in 26.66%, 18.88% and 17.22% of of sheep, goats and cattle herds, respectively (Kaoud *et al.*, 2010). By ELISA, the seroprevalences of brucellosis among livestock and humans in western mountains region in Libya was 31% (goats), 42% (cattle) and 40% (humans) in 2008 (Ahmed *et al.*, 2010).

PCR assay with primers derived from the 16S rRNA sequence for detection of *Brucella* DNA were used by Romero *et al.* (1995). In the past years, a new real-time PCR assay, which combines rapid *in vitro* amplification and quantification of DNA, has been applied to a broad spectrum of infections (Navarro *et al.*, 2004). Real-time PCR assay turned out to be a quick and effective tool for the detection and differentiation of bacterial species and other pathogens in clinical samples (LaGier *et al.*, 2004). Recently, Bounaadja *et al.* (2009) compared real-time PCR and conventional PCR using the same genes. In their research, three genes from *Brucella,* including *IS711*, *bcsp31* and *per* genes were evaluated with both techniques. It was concluded that real-time PCR assays were easy to use, produce results faster than conventional PCR systems while reducing DNA contamination risks. This was confirmed by the results of current study too.

Newby *et al.* (2003) evaluated 3 real-time methods including SYBR Green I (a double-stranded DNA intercalating dye),

5-exonuclease (enzymatically released fluors), and hybridization probes (fluorescence resonance energy transfer) for detection of *B. abortus*. It was found that all three assays were of comparable sensitivity, providing a linear assay over 7 orders of magnitude. The greatest specificity was achieved with the hybridization probe assay.

Surucuoglu *et al.* (2009) tested the advantages of TaqMan real-time PCR technique and compared it to conventional methods using serum samples from patients with different clinical forms of brucellosis. This research showed the high sensitivity and specificity of real-time PCR method and affirmed it as a useful tool for diagnosis of brucellosis with different clinical manifestations.

These studies showed that real-time PCR assay is more sensitive, specific and faster technique than serological and conventional PCR methods for differentiation between *B. abortus* and *B. melitensis*. Although six different species of *Brucella* were recognized, all these species show a high degree of genetic similarity. Therefore, conventional PCR technique, most often, is not able to make a distinction between *Brucella* species. In comparison to real-time PCR analysis, the conventional methods for detecting *Brucella* spp. are technically time-consuming and labourous. The real-time PCR assay used in this study allowed the correct identification of two *Brucella* species (*B. abortus* and *B. melitensis*) and could simplify the procedure by testing presumptive *Brucella* genome taken directly from bovine blood samples. The TaqMan probe offers a specificity higher than that of gel electrophoresis. In addition, this real-time PCR substantially decreases the risk of carryover contamination.

In conclusion, the results of present

study suggest that real-time PCR was highly sensitive and specific for identification and differentiation of *B. melitensis* and *B. abortus* and that it could be a useful tool for diagnosis of brucellosis.

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REFERENCES

Ahmed, M. O., S. E. Elmeshri, A. R. Abuzweda, M. Blauo, Y. M. Abouzeed, A. Ibrahim, H. Salem, F. Alzwam, S. Abid, A. Elfahem & A. Elrais, 2010. Seroprevalence of brucellosis in animals and human populations in the western mountains region in Libya, December 2006–January 2008. *Eurosurveillance*, **15**, 1−3.

Aulakh, H. K., P. K. Patil, S. Sharmma, H. Kumar, V. Mahajan & K. S. Sandhu, 2008. A study on the epidemiology of bovine brucellosis in Punjab (India) using milk-ELISA. *Acta Veterinaria Brno*, **77**, 393−399.

Bogdanovich, T., M. Skurnik, P. S. Lubeck, P. Ahrens & J. Hoorfar, 2004. Validated 5' nuclease PCR assay for rapid identification of the genus *Brucella*. *Journal of Clinical Microbiology*, **42**, 2261−2263.

Bokaie, S., S. Heydari Latibari, S. Abbaszadeh, H. Mousakhani, M. Rabbani & L. Sharifi, 2009. Ecological study of brucellosis in humans and animals in Khoy, a mountainous district of the IR. of Iran. *Iranian Journal of Microbiology*, **1**, 14−17.

Bounaadja, L., D. Albert, B. Che nais, S. Henault, M. S. Zygmunt, S. Poliak & B. Garin-Bastuji, 2009. Real-time PCR for identification of *Brucella* spp.: A comparative study of *IS711*, *bcsp31* and *per* target genes. *Veterinary Microbiology*, **137**, 156−164.

Bricker, B. J., 1999. Differentiation of hard-to-type bacterial strains by RNA mismatch cleavage. *Biotechniques*, **27**, 321−324.

Bricker, B. J., 2002. PCR as a diagnostic tool for brucellosis. *Veterinary Microbiology*, **90**, 435−446.

Bricker, B. J. & S.M. Halling, 1994. Differentiation of *Brucella abortus* bv 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv 1 by PCR. *Journal of Clinical Microbiology*, **32**, 2660−2666.

Carver, T. J., K. M. Rutherford, M. Berriman, M. A. Rajandream, B. G. Barrell & J. Parkhill, 2005. ACT: The Artemis comparison tool. *Bioinformatics*, **21**, 3422−3423.

Foster, J. T., R. T. Okinaka, R. Svensson, K. Shaw, B. K. De, R. A. Robison, W. S. Probert, L. J. Kenefic, W. D. Brown & P. Keim, 2008. Real-time PCR assays of single-nucleotide polymorphisms defining the major *Brucella* clades. *Journal of Clinical Microbiology*, **46**, 296−301.

Gul, S. T. & A. Khan, 2007. Epidemiology and epizootology of brucellosis: A review. *Pakistan Veterinary Journal*, **27**, 145−151.

Kaoud, H. A., M. M. Zaki, A. R. El-Dahshan & S. A. Nasr, 2010. Epidemiology of brucellosis among farm animals. *Nature and Science*, **8**, 190−197.

Kazemi, B., S. A. Yousefi Namin, M. Dowlatshahi, M. Bandepour, F. Kafilzadeh, L. Gachkar, F. Mahmoudinejad, A. Samarghandi & M. Mardani, 2008. Detection of *Brucella* by peripheral blood PCR and comparison with culture and serological methods in suspected cases. *Iranian Journal of Public Health*, **37**, 96−102.

LaGier, M. J., L. A. Joseph, T. V. Passaretti, K. A. Musser & N. M. Cirino, 2004. A real-time multiplexed PCR assay for rapid detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli*. *Molecular and Cellular Probes*, **18**, 275−282.

Leal-Klevezas, D., I. O. Martinez Vazquez, A. Lopez-Merino & J. P. Martinez-Soriano, 1995. Single-step PCR for detection of *Brucella* spp. from blood and milk of in-

fected animals. *Journal of Clinical Microbiology*, **33**, 3087−3090.

Moradi, G., N. Esmaiel Nasab, E. Ghaderi, M. Sofi Majidpour & H. Salimzadeh, 2006. Brucellosis in Kurdistan province from 1997 to 2003. *Annals of Alquds Medicine*, **2**, 32−37.

Navarro, E., M. A. Casao & J. Solera, 2004. Diagnosis of human brucellosis using PCR. *Expert Review of Molecular Diagnostics*, **4**, 115−123.

Newby, D. T., T. L. Hadfield & F. F. Roberto, 2003. Real-time PCR detection of *Brucella abortus*: A comparative study of SYBR green I, 5′-exonuclease, and hybridization probe assays. *Applied and Environmental Microbiology*, **69**, 4753−4759.

Otlu, S., M. Sahin, H. I. Ataba & A. Unver, 2008. Serological investigations of brucellosis in cattle, farmers and veterinarians in the Kars District of Turkey. *Acta Veterinaria Brno*, **77**, 117−121.

Probert, W. S., K. N. Schrader, N. Y. Khuong, S. L. Bystrom & M. H. Graves, 2004. Real-time multiplex PCR assay for detection of *Brucella* spp., *B. abortus*, and *B. melitensis*. *Journal of Clinical Microbiology*, **42**, 1290−1293.

Refai, M., 2000. Control of brucellosis in animals in Egypt. In: *Proceedings of the 2nd International Symposium cum-Workshop of the Germany-Egypt-Region Inter-Alumni-Net (GEAR)*, St. Catherine, Germany.

Refai, M., 2002. Incidence and control of brucellosis in the Near East region. *Veterinary Microbiology*, **90**, 81−110.

Romero, C., C. Gamazo, M. Pardo & I. Lopez-Goni, 1995. Specific detection of *Brucella* DNA by PCR. *Journal of Clinical Microbiology*, **33**, 615−617.

Sahin, M., A. Unver & S. Otlu, 2008. Isolation and biotyping of *Brucella melitensis* from aborted sheep foetuses in Turkey. *Bulletin of the Veterinary Institute in Pulawy*, **52**, 59−62.

Sambrook, J. & D. W. Russell, 2001. Molecular Cloning: A Laboratory Manual. 3rd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Surucuoglu, S., S. El, S. Ural, H. Gazi, S. Kurutepe, P. Taskiran & S. G. Yurtsever, 2009. Evaluation of real-time PCR method for rapid diagnosis of brucellosis with different clinical manifestations. *Polish Journal of Microbiology*, **58**, 15−19.

Tahamtan, Y., M. M. Namavari, G. Mohammadi & G. Moazeni Jula, 2010. Prevalence of brucellosis in horse North-East of Iran. *Journal of Equine Veterinary Science*, **30**, 376−378.

Tcherneva, E., N. Rijpens, B. Jersek & L. M. Herman, 2000. Differentiation of *Brucella* species by random amplified polymorphic DNA analysis. *Journal of Applied Microbiology*, **88**, 69−80.

Zvizdic, S., D. Cengic, M. Bratic, S. Mehanic, F. Pinjo & S. Hamzic, 2006. *Brucella melitensis* review of the human infection case. *Bosnian Journal of Basic Medical Sciences*, **6**, 15−18.

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