International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:5, No:2, 2011

The First Prevalence Report of Direct Identification and Differentiation of *B. abortus* and *B. melitensis* using Real Time PCR in House Mouse of Iran

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***Abstract*—**Brucellosis is a zoonotic disease; its symptoms and appearances are not exclusive in human and its traditional diagnosis is based on culture, serological methods and conventional PCR. For more sensitive, specific detection and differentiation of *Brucella* spp., the real time PCR method is recommended.

This research has performed to determine the presence and prevalence of *Brucella* spp. and differentiation of *Brucella abortus* and *Brucella melitensis* in house mouse (*Mus musculus*) in west of Iran. A TaqMan analysis and single-step PCR was carried out in total 326 DNA of Mouse's spleen samples. From the total number of 326 samples, 128 (39.27%) gave positive results for *Brucella* spp. by conventional PCR, also 65 and 32 out of the 128 specimens were positive for *B. melitensis*, *B. abortus*, respectively.

These results indicate a high presence of this pathogen in this area and that real time PCR is considerably faster than current standard methods for identification and differentiation of *Brucella* species. To our knowledge, this study is the first prevalence report of direct identification and differentiation of *B. abortus* and *B. melitensis* by real time PCR in mouse tissue samples in Iran.

***Keywords*—**Differentiation, *B. abortus*, *B. melitensis*, TaqMan probe, Iran.

I. Introduction

BRUCELLOSIS is an important public health problem in many parts of the worlds, such as the Mediterranean littoral, the Middle East and parts of Latin America [1]. Brucellosis is a zoonotic disease that caused by genus *Brucella*. This disease in humans causes fever, malaise, myalgia and may later develop into a chronic illness affecting various organs and tissues. Also in animals, causes abortion, fetal death, and genital infections [2]. There are various methods for diagnosis of brucellosis such as culture, serological and molecular methods. Culture methods are well established for brucellosis but highly dangerous to laboratory workers, difficult and lengthy process that requires experienced technicians. Often taking 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 [3]. The serological methods usually employed for diagnosis *Brucella* in blood specimens. The diagnosis of brucellosis by serological responses, which can be unspecific due to cross-reaction or subsensitive reactions in samples from areas with a low or subclinical prevalence of brucellosis [4]. Therefore, molecular techniques such as PCR and real-time PCR (rtPCR) that are simpler, faster, less hazardous and usually more sensitive have been developed for *Brucella* detection [5].

Although several *Brucella* genus specific assays have been described, none of them differentiate between different species [6]. 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 very time consuming [7], [8].

Many molecular methods are available for differentiation of six *Brucella* species on the basis of size of PCR product, PCR-RFLP, RAPD analysis, etc. [9], [10], [11]. At the present time, there are several conventional and real-time PCR assays for differentiation between *Brucella* species [12].

The aim of this study was to determine the presence and prevalence of *Brucella* spp. and differentiation of *B. abortus* and *B. melitensis* in house mouse (*Mus musculus*) in west of Iran.

II. Materials and Methods

Sample and DNA extraction

A total of 326 house mouse were collected in 3 provinces (Isfahan [n=145], Chaharmahal Va Bakhtiari [n=99] and Lorestan [n=82]) located in west of Iran. Mice were collected at summer of 2009, placed in separate sterile plastic bags to prevent spilling and cross contamination, and immediately transported to the laboratory in an ice box.

The spleens were isolate from mice and purification of DNA was achieved using a genomic DNA purification kit (Invitrogen, Paisley, U.K.) according to the manufacturer's instructions. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell [13].

Conventional PCR assay

PCR primers that used to screen the *Brucella* spp. was detected DNA sequence of the gene coding for the outer membrane protein (omp-2) reported for *Brucella* in GenBank database located at NCBI [14]. The forward primer sequence is 5'-GCGCTCAGGCTGCCGACGCAA-3', and the reverse primer sequence is 5'-ACCAGCCATTGCGGTCGGTA-3'.

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All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran).

The PCR reaction was performed in a total volume of 25 µL containing 2 µL of DNA sample, 0.5 mm $MgCl_2$, 0.2 mm dNTP, 0.8 µm each primers 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 50 s, 65°C for 40 s, 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 (Master Cycler Gradiant, Eppendrof, Germany). A negative control (sterile water), and a positive control DNA from *B. abortus* strain S19 (vaccine strain), 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 (0.4µg/mL) and viewed on UV transilluminator.

Real time PCR assay

The Real time PCRs for species differentiation were based on unique genetic loci of *B. melitensis* and *B. abortus*. The regions were chosen for the construction of primers and TaqMan® probes for species differentiation: BMEII0466 gene for *B. melitensis* and BruAb2_0168 gene for *B. abortus* (Table I). A typical 25 µL reaction contained: 12.5 µl TaqMan® Universal PCR Master Mix (Roche), a 300 nM concentration of each forward and reverse primer (BioNeer Corporation, South Korea), a 200 nM concentration of the probe (BioNeer Corporation, South Korea), and 2.5 ng of sample DNA. TaqMan Master Mix Real time PCRs reactions were carried out using a RotorGene 6000 instrument (Corbett Research). The reaction mixture was initially incubated for 10 min at 95°C. Amplification was performed for 45 cycles of denaturation at 95°C for 20 s, annealing and extension at 62°C for 1 min.

TABLE I
REAL TIME PCR PRIMERS AND TaqMan® PROBES

Target sequence	primers (5′→3′)	Probe (5′Fluorophore→3′Quencher)	Fragment size
BMEII0466	TCGCATCGGCAGTTTCAA / CCAGCTTTTGGCCTTTTCC	Cy5-CCTCGGCATGGCCCGCAA-BHQ-2	112bp
BruAb2_0168	GCACACTCACCTTCCACAACAA / CCCCGTTCTGCACCAGACT	FAM-TGGAACGACCTTTGCAGGCGAGATC-BHQ-1	222bp

III. Results

In this study, a total of 326 spleen samples of house mouse from three 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 product showed the presence of bands of 113-bp fragment for *Brucella* spp. The presence of *Brucella* DNA was detected by single PCR in spleen samples were from 128 out of 326 animals (39.27%). Molecular results indicate that the prevalence of Brucella spp in house mice from each province is shown in table II.

TABLE II
DISTRIBUTION OF *B. MELITENSIS*, *B. ABORTUS* AND *BRUCELLA* SPP. IN WEST OF IRAN

Province	No. of samples	Conventional PCR assay (%)	Real time PCR assay (%)			
			B. melitensis	*B. abortus*	Unknown	both bacteria
Isfahan	145	48 (33.10)	22 (15.17)	13 (8.96)	13 (8.96)	3 (2.06)
Chaharmahl va Bakhtiari	99	43 (43.43)	25 (25.25)	8 (8.08)	10 (10.10)	5 (5.05)
Lorestan	82	37 (45.12)	18 (21.95)	11 (13.41)	8 (9.75)	4 (4.87)
Total	326	128 (39.27)	65 (19.93)	32 (9.81)	31 (9.50)	12 (3.68)

After real time PCR, BMEII0466 and BruAb2-0168 gene were distinguished in 65, 32 and 12 out of the 128 specimens were positive for *B. melitensis*, *B. abortus* and both bacteria, respectively. On the other hand, by using of BMEII0466 and BruAb2-0168 gene specific primers, none of two species (*B. melitensis* and *B. abortus*) found in 31 samples (Table 2). Figures show a typical example of conventional real time PCR results for representative isolates of *Brucella* spp. and differentiation of *B. melitensis* and *B. abortus* (Fig.1 and 2).

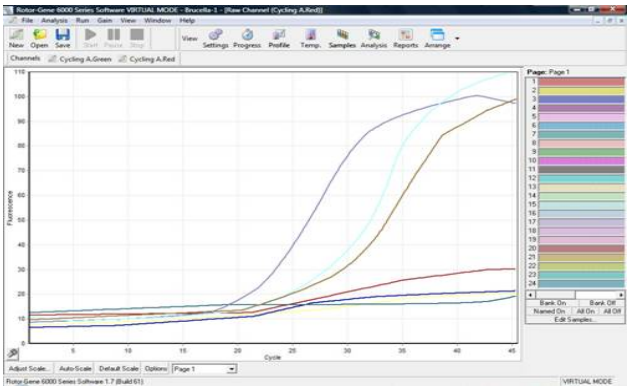

Fig. 1 Graph obtained by real time PCR from red channel for identification of *B. abortus*. CT less than 43 were indicating as positive

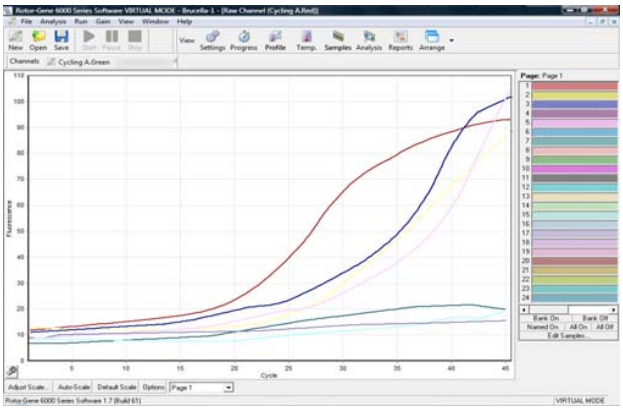

Fig. 2 Graph obtained by real time PCR from green channel for identification of B. melitensis CT less than 43 were indicating as positive

IV. Discussion

The genus *Brucella* comprises six classical species that important agents of human disease are *B. melitensis*, *B. abortus* and *B. suis* [15]. The transmission of *Brucella* infection and its prevalence in a region depends upon several factors like food habits, methods of processing milk and milk products, social customs, husbandry practices, climatic conditions, socioeconomic status and environment hygiene [16]. Humans are commonly infected through ingestion of raw milk, cheese and meat or through direct contact with infected animals [17].

Brucellosis is almost invariably transmitted to human from infected animals such as ruminant, some species of mice and mouselike rodents [18]. Therefore, the mice play an important role in *Brucella* transmission to humans and domestic animals.

Romero et al. in 1995 used PCR assay with primers derived from the 16S rRNA sequence for detection of *Brucella* DNA [19].

In the past few 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 [20]. Also real time PCR assay could be a valuable tool for the detection and differentiation of bacterial species in clinical samples [21].

Recently, Bounaadja et al. was compared the real time PCR assays and conventional PCR using the same genes. In their research, three genes from *Brucella* including IS711, bcsp31 and per genes with both techniques were evaluated. They concluded, real-time PCR assays are easy-to-use, produce results faster than conventional PCR systems while reducing DNA contamination risks [22].

The prevalence of total *Brucella* observed in mice in this study (9.3%) is in agreement with those recently reported in mice samples from India (Raghonath 2008) and Malaysia (Sujeewa et al., 2009); however, is lower than the results reported from Thailand (75.8%) (Wong et al., 1999), Taiwan (70.2%) (Wong et al., 1992), and China (39.4%) (Yang et al., 2008). Our results is higher than that reported from Turkey (0.8%) (Colakoglu et al., 2006) and from one previously reported from Iran (2.1%) (Hosseini et al., 2004).

Although according to some features the six different species of *Brucella* were recognized, all of these species show high degrees of genetic similarity. Therefore, conventional PCR technique, most often, is not able to precise distinction between *Brucella* species. The results of this study indicate that the conventional PCR technique is often able to correctly identify *Brucella* spp. In addition, in comparison to real time PCR analysis, the conventional methods for detecting *Brucella* spp. are technically time-consuming and labor-intensive.

The real time PCR assay that use in this study allows correct identification of two *Brucella* species (*B. abortus* and *B. melitensis*) and can simplify the procedure by testing presumptive *Brucella* genome taken directly from mice tissue. Using the TaqMan probe offers specificity higher than that of gel electrophoresis. In addition, this real time PCR can substantially decrease the risk of carryover contamination.

To our knowledge, this study is the first report of direct identification and differentiation of *Brucella* spp by real time PCR in mice tissue samples (spleen) in Iran. Further intensive prevalence studies on *Brucella* infection among house mouse will be needed to elucidate the epidemiology of brucellosis in Iran.

Acknowledgment

We would like to thank head and deputy of research of Islamic Azad University of Shahrekord branch in Iran for their sincere support.

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