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MOLECULAR STUDY OF BRUCELLOSIS IN CAMELS BY THE USE OF TaqMan® REAL-TIME PCR

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Brucellosis is a zoonosis of economic importance that reduces productivity in livestock enterprises as it induces abortion in infected animals. A study was designed aimed at detecting *Brucella* in blood and lymph node specimens from camels by the use of real-time PCR in Iran. Sample collection and DNA extraction were done on blood (n = 135) and lymph node (n = 135) samples collected from 135 camels (abattoir survey) from both sexes at various ages in different seasons. The real-time PCR for species differentiation was 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 Bru-Ab2_0168 gene for *B. abortus*. *Brucella* spp. were identified in 18 (13.33%) blood samples and 4 (2.97%) lymph node samples. This method showed to be effective in detecting *B. abortus* and *B. melitensis* in blood and lymph samples respectively. *Brucella abortus* was detected in 3 (2.22%) blood samples but was however, not detected in the lymph node samples. *Brucella melitensis* was only observed in 4 (2.97%) lymph node samples. Significant differences were observed on the blood prevalence of unknown *Brucella* spp. in different age groups and seasons ($P < 0.05$). However, there were no significant differences observed on the prevalence of *B. abortus, B. melitensis,* unknown *Brucella* spp. in different age groups, sex and seasons ($P > 0.05$). Therefore, *Brucella* was detected in apparent healthy camels slaughtered at an abattoir in Iran and this recommends the significance of the detection of *Brucella* in camels, since the infected camels appear to be healthy.

Keywords: camel, *Brucella*, blood, lymph node, real-time PCR

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Introduction

Brucellosis is a severe zoonosis caused by bacteria of the genus *Brucella* and is a public health problem in many parts of the world, and common in Arabian region, Latin America, Africa, and some parts of Asia like Iran [1]. *Brucella* genus infects a wide range of animal hosts including camels, cows, goats, sheep, pigs, bison, dogs, elks, and even marine mammals. The genus *Brucella* currently comprises ten species: *B. melitensis*, *B. suis*, *B. abortus*, *B. ovis*, *B. canis*, *B. neotomae*, *B. ceti, B. microti, B. pinnipedialis,* and *B. inopinata* [2, 3]. Investigations from 16S rRNA gene sequence chemical analysis and other biochemical characteristics suggested that the *Brucella* spp. comprise a monophyletic genus [4].

Laboratory diagnostic techniques for brucellosis mainly rely on serological tests that detect antibodies against *Brucella* and cultivation of blood or tissue cultures [5]. However, research developed significant DNA diagnostic techniques for brucellosis that utilize the selectivity and sensitivity of PCR. Targeted gene has included 16S–23S spacer regions [6], 16S rRNA gene sequences [7], outer membrane proteins [8], erythritol utilization genes [9], and insertion sequences [10]. The assays have been applied to bacterial isolates, clinical specimens and blood [11]. Moreover, single primer sets and multiplexed assays [12] with a combination of primers allow the detection of most of the biovars of *Brucella* by conventional PCR.

Camels are susceptible to both *B. abortus* and *B. melitensis* but are not identified to be primary hosts of *Brucella* [13]. In Iran camels are a division of enormous livestock possessions. The major part of the country is made up of arid areas that are suitable for camel production. As a consequence, camel husbandry is widely practiced. There is however, limited information on the application of real-time PCR for detection of *Brucella* in blood and lymph specimens in camels. Therefore, a study was designed with the aim of detecting *Brucella* spp. in blood and lymph node specimens by the use of real-time PCR in camels in Iran.

Materials and Methods

Sampling

Sample collection and DNA extraction were done on a total of 270 samples (from 135 blood and 135 lymph node samples). Blood (n = 135) and lymph node (n = 135) samples were collected over a period of 12 months (May 2013 to May 2014) from 135 camels (abattoir survey) from both sexes at various ages in different seasons in Iran. The camels *(Camelus dromedaries)* were apparently healthy

at the time of slaughter and none had been previously immunized against *Brucella* spp. (the camels were not serologically tested for brucellosis). All samples were collected under sterile hygienic conditions. From each animal, approximately 8 to 10 millilitres of the whole blood (with anticoagulant) was aseptically taken and was used for real-time PCR. After slaughtering the animals, lymphoid tissues were sampled from the subscapular lymph nodes and immediately placed in a sterile container. All samples were kept on ice and transported to the Biotechnology Research Centre of Islamic Azad University of Shahrekord laboratory. Blood and lymph tissue samples were kept frozen (−20 °C) until analysis.

DNA extraction from blood and lymph tissue samples

DNA was extracted using a genomic DNA extraction kit (DNP™, CinnaGen, Tehran, Iran) according to the manufacturer's recommendation. The total DNA was measured at 260 nm wavelength according to the method described by Sambrook and Russell [14]. Distilled water instead of template DNA was routinely used as negative control in each PCR together with the DNA samples to avoid contamination. Positive controls with genomic DNA of *Brucella* were included in each run to detect any amplicon contamination or amplification failure. Samples were then loaded onto a 10% polyacrylamide gel in 0.5X TBE for electrophoresis at 150 V for 5 h. After the electrophoresis run, bands were silver stained according to Bassam et al. [15].

Real-time PCR assay

The real-time PCR for species differentiation was 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 tube contained: 12.5 µL TaqMan® Universal PCR Master Mix (Roche Applied Science, Indianapolis, IN, USA), a 300 nM concentration of each forward and reverse primer (BioNeer Corporation, South Korea), a 200 nM concentration of the probes labelled 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 instrument (Corbett Research, Sydney, Australia). 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 and 72 °C, respectively, for 20 s. The cycle

Table I. Primers and TaqMan® probes used in this study

Target sequence	Forward primer/reverse primer (5′→3′)	Probe (5′Fluorophore→3′Quencher)	Fragment size	Reference
IS711	GCTTGAAGCTTGCGGACAGT/ GGCCTACCGCTGCGAAT	FAM-AAGCCAACACCCGGCCATTATGGT-TAMRA	63 bp	[16]
BMEII0466	TCGCATCGGCAGTTTCAA/ CCAGCTTTTGGCCTTTTCC	Cy5-CCTCGGCATGGCCCGCAA-BHQ-2	112 bp	
BruAb2_0168	GCACACTCACCTTCCACAACAA/ CCCCGTTCTGCACCAGACT	FAM-TGGAACGACCTTTGCAGGCGAGATC-BHQ-1	222 bp	

threshold (Ct) value was calculated for each sample as the number of cycles at which fluorescence exceeded the threshold limit, which was set at the top of the second derivative fluorescence curve and expressed as fractional cycle numbers. Ct values ranged were considered from 18 to 30 Ct. Detection of fluorescence over 35 cycles was considered as indicative of a false-positive.

Statistical analysis

Data were analyzed using the SPSS/20.0 software and the P-value was calculated using the Chi-square and Fisher's exact tests. Statistical significance was regarded at a P-value < 0.05.

Results

A total of 135 (100 males, 35 females and 108 adult, 27 young) camels were tested and 270 equal blood and lymph node samples were analysed. Table II shows the distribution of *Brucella* spp., *B. melitensis* and *B. abortus* in the samples collected from the camels. Of the total (270) samples tested, 22 (8.15%) had *Brucella* spp., with 3 (2.22%) of them having *B. abortus,* 4 (1.49%) having *B. melitensis* and 15 (11.11%) being unknown. *Brucella* spp. were identified in 18 (13.33%) blood samples and 4 (2.97%) lymph node samples. *Brucella abortus* was detected in 3 (2.22%) blood samples but was however, not detected in the lymph node samples. *Brucella melitensis* was only observed in 4 (2.97%) lymph node samples. The unknown 15 (11.11%) samples were only observed in the blood samples. Significant differences were observed on the blood prevalence of unknown *Brucella* spp. in different age groups and seasons ($P < 0.05$). There were no significant differences observed on the prevalence of *B. abortus, B. melitensis, Brucella* spp. in different age groups, sex and seasons ($P > 0.05$). *Brucella abortus* and *B. melitensis* can be effectively detected in blood and lymph node samples, respectively. More *Brucella* was observed in samples collected from adult camels as compared to those from young camels. Samples collected in the summer had the largest amount of *Brucella* compared to those from the other seasons. There was a high *Brucella* prevalence in samples collected from female camels than in samples from male camels. Figures 1, 2 and 3 show real-time PCR amplification curves of the *Brucella* genus, *B. melitensis* and *B. abortus,* respectively.

Table II. Distribution of *Brucella* spp., *B. abortus* and *B. melitensis* in camel samples by real-time PCR

Risk factors	Number of samples		*Brucella* spp. (%)	*B. abortus (%)*	*B. melitensis (%)*	*Unknown (%)*	*Both bacteria (B. abortus + B. melitensis) (%)*
				Samples			
Blood	135		18 (13.33)	3 (2.22)	0 (0)	15 (11.11)	0 (0)
Lymph node	135		4 (2.97)	0 (0)	4 (2.97)	0 (0)	0 (0)
Total	270		22 (8.15)	3 (2.22)	4 (1.49)	15 (11.11)	0 (0)
				Sex			
Male	100	*B*	12 (12)	3 (3)	0 (0)	9 (9)	0 (0)
		L	3 (3)	0 (0)	3 (3)	0 (0)	0 (0)
Female	35	*B*	6 (17.14)	0 (0)	0 (0)	6 (17.14)	0 (0)
		L	1 (2.86)	0 (0)	1 (2.86)	0 (0)	0 (0)
Total	135	*B*	18 (13.33)	3 (2.22)	0 (0)	15 (11.11)	0 (0)
		L	4 (2.97)	0 (0)	4 (2.97)	0 (0)	0 (0)
				Age			
Adult	108	*B*	17 (15.74)	2 (1.85)	0 (0)	15 (13.88)	0 (0)
		L	4 (3.7)	0 (0)	4 (3.7)	0 (0)	0 (0)
Young	27	*B*	1 (3.7)	1 (3.7)	0 (0)	0 (0)	0 (0)
		L	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	135	*B*	18 (13.33)	3 (2.22)	0 (0)	15 (11.11)	0 (0)
		L	4 (2.97)	0 (0)	4 (2.97)	0 (0)	0 (0)
				Seasonal			
Summer	40	*B*	11 (27.5)	2 (5)	0 (0)	4 (10)	0 (0)
		L	3 (7.5)	0 (0)	1 (2.5)	0 (0)	0 (0)
Autumn	30	*B*	4 (13.33)	0 (0)	0 (0)	4 (13.33)	0 (0)
		L	0 (0)	0 (0)	2 (6.67)	0 (0)	0 (0)
Winter	25	*B*	2 (8)	0 (0)	0 (0)	3 (12)	0 (0)
		L	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Spring	40	*B*	1 (2.5)	1 (2.5)	0 (0)	4 (10)	0 (0)
		L	1 (2.5)	0 (0)	1 (2.5)	0 (0)	0 (0)
Total	135	*B*	18 (13.33)	3 (2.22)	0 (0)	15 (11.11)	0 (0)
		L	4 (2.97)	0 (0)	4 (2.97)	0 (0)	0 (0)

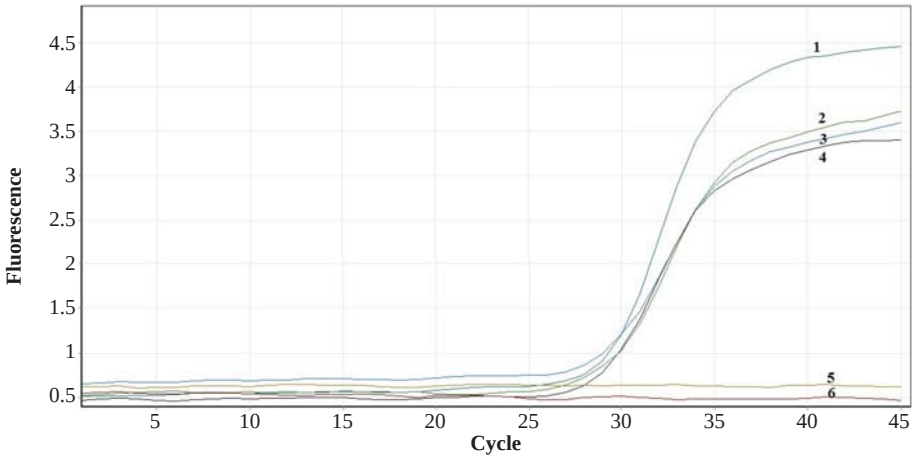

Figure 1. Real-time PCR amplification curves of the *Brucella* genus
1) Positive control of *Brucella;* 2, 3 and 4) Positive sample; 5) Negative control; 6) Negative sample

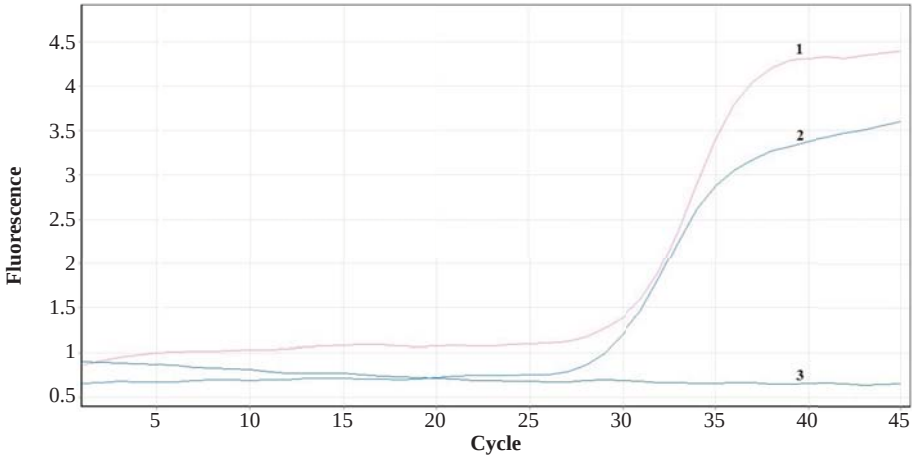

Figure 2. Real-time PCR amplification curves of the *Brucella abortus*
1) Positive control of *Brucella abortus;* 2) Positive sample; 3) Negative control

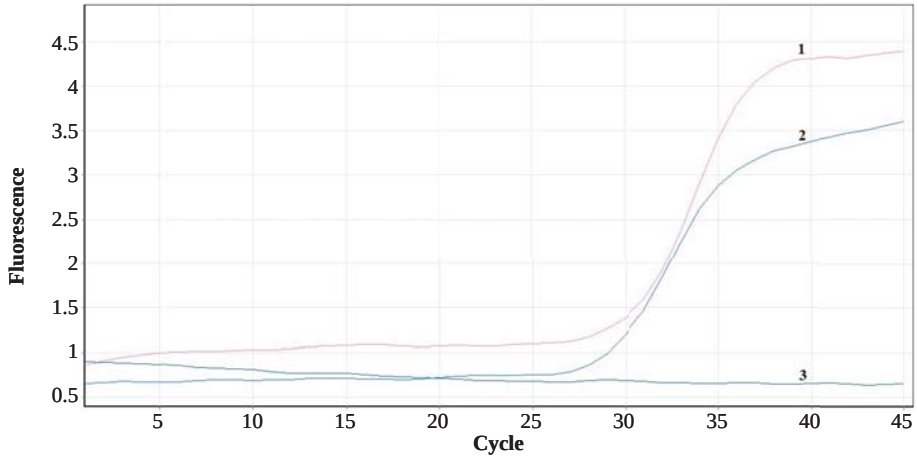

Figure 3. Real-time PCR amplification curves of the *Brucella melitensis*
1) Positive control of *Brucella melitensis;* 2) Positive sample; 3) Negative control

Discussion

The purpose of this study was to describe real-time PCR assay for the detection of *Brucella* spp. in Iran. The real-time PCR assay is more sensitive compared to the rapid immunological methods. Moreover, with immunological procedures antigen-antibody interactions can be difficult by nonspecific interactions and false positives from vaccinated animals with high levels of circulating antibodies can be observed [17]. The results of the present study showed that apparent healthy camels can be an important reservoir for transmission of these zoonotic diseases to humans in Iran.

Radwan et al. [18] and the World Health Organisation [19] reported the level of prevalence of *B. melitensis* and *B. abortus* in camels that support the results observed in this study. Zowghi and Ebadi [20] isolated *B. melitensis* in several camels in Iran, whereas in Sudan, where camels were reared together with goats, cattle and sheep, Agab et al. [21] observed *B. abortus* from lymph nodes of camels. However, in this study no *B. abortus* was detected in the lymph node samples.

A higher prevalence of *Brucella* spp. of 18 (13.33%) and 4 (2.97%) from blood and lymph node samples respectively, was observed in the current study in comparison to previous studies [22–24]. The high prevalence of *Brucella* detected by PCR in female as compared to male camels in this research is in agreement with previous studies on seroprevalence by Teshome et al. [22], Tefera [23], Warsame et al. [24] and Khamesipour et al. [25]. The observed results may

be due to decreased immunity in females due to reproductive stress, lactation, and pregnancy. According to Radostitis et al. [26], behavioural and physiological differences between female and male animals result in the difference in sex susceptibility to brucellosis.

The large dominance of *Brucella* observed in adult as opposed to young camels is in agreement with the work of Tefera [23] and Warsame et al. [24]. This can be attributed to sex hormones that have a propensity to increase in concentration with age and sexual maturity and promote growth and multiplication of *Brucella* [26]. Additionally, this is supported by Gyles and Prescott [27] who elucidated that younger animals are more resistant to infection and frequently clear established infection compared to older animals. However, Khamesipour et al. [25] revealed opposite findings: young camels were the most commonly infected age group, while adult camels were the less often infected age group.

Studies illustrated that *B. abortus* and *B. melitensis* constitute the majority of *Brucella* species that are frequently detected in clinical specimens of diseased camels [28, 29]. However, in this study most of the *Brucella* spp. detected was not *B. abortus* and *B. melitensis.* Also, in this study more *Brucella* spp. was detected in blood compared to lymph specimens. Abbas and Agab [30] revealed that blood cultures set for the "gold standard" of laboratory diagnosis and positive blood cultures contain 10% to 70% of suspected infections and this depends on the duration, localization of the infection and the type of *Brucella* species. Khamesipour et al. [25] observed 4.07% and 2.44% camel blood samples were positive for *B. abortus* and *B. melitensis*, respectively, whilst 3.25% and 1.63% lymph node samples were positive for *B. abortus* and *B. melitensis*, respectively.

In the present study, an overall prevalence of 8.15% was recorded in camels using the real-time PCR. This finding is higher than that obtained by Gameel et al. [31] who recorded a prevalence of 4.1% in Libya and Teshome et al. [22] who observed prevalence of 4.2% in Borena. Bekele [32] reported much lower values 0.4 to 2.5% in Borena. In addition, the prevalence in this study is higher than that recorded by Teshome et al. [22] and Zewolda and Wereta [33] with prevalence of 5.5% and 5.7%, respectively. However, Zewolda and Wereta [33] observed almost the same prevalence 7.6% in the Afar region. It is also within the range of 6.0% to 38.0% reported by Wilson et al. [34] in Kenya and by Osman and Adlam [35] in Sudan. The differences may perhaps be due to the disparity in agro-ecology and sample size used. Additionally, the discrepancies could also be attributed to variations in animal husbandry and production systems. However, Abbas and Agab [30] substantiated the differences in prevalence of brucellosis in camels to follow two discrete outlines: high (8–15%) prevalence in camels kept intensively or semi-intensively and low (2–5%) prevalence in nomadic or extensively kept camels.

Moreover, the differences in brucellosis prevalence observed in this study compared to other studies may be attributed to the variation in the tests used. None of the regularly used serological test can be alleged as a perfect test for *Brucella* detection in camels and the majority of serological tests applied for camels have been directly transposed from cattle lacking adequate validation, as a result an inaccurate diagnosis might arise when diagnosis is based on serology alone [36]. Results reported by Queipo-Ortuno et al. [37] showed that real-time PCR applied to serum samples was more sensitive than other methods. A study by Gwida et al. [36] showed that combination of real-time PCR with one of the conventional serological tests can identify brucellosis in more than 99% of the infected animals. According to Yu and Nielsen [38] the major advantages of real-time PCR are that it can be performed in a very short time, with no requirement for electrophoretic analysis, and circumvent contamination.

Conclusions

The study indicated that brucellosis is a potential disease in apparent healthy camels in Iran. Thus, persons in contact with camels ought to be cautious of the possibility of camels being a source of brucellosis.

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Conflict of Interest

No conflict of interest.

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