

## MOLECULAR STUDY OF THE PREVALENCE OF *BRUCELLA ABORTUS* AND *BRUCELLA MELITENSIS* IN THE BLOOD AND LYMPH NODE SAMPLES OF SLAUGHTERED CAMELS BY POLYMERASE CHAIN REACTION (PCR) IN IRAN

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Brucellosis is a zoonotic disease which is characterized by reduced fertility and abortion in several species of animals, as well as humans. Camel brucellosis is caused by *Brucella abortus* and *Brucella melitensis*. To overcome the limitations posed by other techniques such as culture and serology, a sensitive technique (PCR) was employed for the detection of brucellosis in 123 camels. Findings from this PCR study indicated a total of 11.38% of blood samples as positive for *Brucella* spp. and 13.01% of the lymph node samples were positive for *Brucella* spp. In this study, 5 out of 123 (4.065%) and 3 out of 123 (2.439%) camel blood samples were positive for *B. abortus* and *B. melitensis*, respectively. Also, 4 out of 123 (3.252%) and 2 out of 123 (1.626%) camel lymph node samples were positive for *B. abortus* and *B. melitensis*, respectively. Young camels were the most commonly infected age group, while adult camels were the less often infected age group. Also, higher prevalence of brucellosis was observed in female camels. These results have indicated that PCR is a sensitive technique which could be used as a confirmatory test for the detection of brucellosis in live camels, at the same time with the lowest risk of infection of laboratory personnel. The obtained results suggest that control and eradication programs for *Brucella* spp. infection seem to be necessary in camels. Our findings support the power of PCR test for *Brucella* spp. detection in the blood and lymph node samples and it could be easily used for routine diagnosis.

**Key words:** camel, brucellosis, zoonosis, blood, lymph nodes, Polymerase Chain Reaction (PCR)

### INTRODUCTION

Camels are the toughest animal species for production and survival under harsh environmental conditions and have been considered an aid to man for thousands

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of years. Camels have a high money-based value by providing meat, milk, wool, as well as transportation and labor. The camel is a domestic mammal which due to its physiological attributes is suitable for use in climatic extremes. Diseases, poor nutrition, and traditional management systems have restricted their full utilization [1,2]. Even though several pastoral groups and communities all over the world depend on camels for their livelihood, the health status of camels has not yet received proper attention from researchers and scientists.

The camel is a domestic animal that may be infected with *Brucella*. Camel brucellosis is caused by Gram-negative coccobacilli bacteria of the genus *Brucella* and is characterized by lesions of lymph nodes and joint capsules, orchitis and epididymitis, inflammation of the uterus, abortion, and reduced fertility. Also, many infected camels are silent carriers of brucellosis [1-3]. Brucellosis remains to be the main zoonosis and is found globally. Furthermore, it seems that the issue of *Brucella* in the camel has potentially important implications for public health and implementation of brucellosis control programs. Primarily, the camel may act as a reservoir for the dissemination of contaminated secretions to other domestic animals and humans. Secondly, in several nations, no formal surveillance and eradication programs for camel brucellosis have been proposed [2,3]. In some developed nations brucellosis is well controlled, however in Africa, Asia, South and Central America, and the Middle East, the clinical disease is still present among individual owners of camels. In Iran, *B. melitensis* and *B. abortus* are an overall public problem [4].

The genus *Brucella* consists of 8 species. Camels are highly susceptible to *Brucella abortus* (*B. abortus*), and *Brucella melitensis* (*B. melitensis*) [1], but camels are not known to be primary hosts of *Brucella*. Thus, camel brucellosis depends on the *Brucella* species prevalent in other animal species sharing the same habitats, and on husbandry methods [1]. Moreover, the main species affecting humans are *B. abortus* and *B. melitensis*, which cause brucellosis, also known as Malta fever [4].

Consumption of *Brucella* infected food e.g. milk and meat from camels has led to a high number of human brucellosis cases and is a serious public health issue. The situation is even more grave as farmers from rural areas think that raw camel milk has a healing effect on the digestive system [1].

The eradication of brucellosis is an essential step to control the disease in humans [2]. Cattle, goat, sheep, camels and other livestock may be infected and transmit the disease to human populations. Moreover, pastoralists in endemic areas are at high risk of infection by *Brucella* species [5]. Brucellosis of camels in Iran has been studied mainly by serological methods, without isolation of the causative agent [2].

Currently, diagnosis of brucellosis is based on serological and microbiological tests. Serological methods are not always sensitive or specific and are laborious (little sensitivity), time-consuming, pose a risk for infection, and can generate discordant results [1,2].

Isolation and identification are the most reliable techniques in the diagnosis of brucellosis, even though not always successful, and represent a major infection

risk for technicians [2]. Microbial culture may be used for several suspected cases, nonetheless is not used for surveying the disease in the camel populations [2,7]. Also, with these two methods, species cannot be differentiated from each other [4]. In consequence, over the last few years progress has been made in applying new molecular and genetic diagnostic methods to improve the diagnosis of brucellosis and nucleic acid amplification techniques might circumvent the diagnostic window being presented before production of specific antibodies [1,2,8]. One of these methods, the polymerase chain reaction (PCR) is a sensitive, fast, and relatively cheap method and is mainly useful in the detection of *Brucella* DNA in tissues and body fluids contaminated with non-viable or a low number of *Brucella* [2]. There are few publications on using PCR in the detection of camel brucellosis [2,7].

The objective of this study was to determine the prevalence of *Brucella* spp. in lymph nodes and blood samples from camels by using PCR method and to identify potential risk factors for infection. PCR is a rapid and simple technique capable of specifically detecting *Brucella* infection in camels.

## MATERIALS AND METHODS

### ***Location and sampling***

The Najaf-Abad region is located in the west of Isfahan province, Center of Iran. Blood (n=123) and lymph node (n=123) samples were collected from 123 camels from both sexes and different ages of camels in Najaf-Abad's abattoir in Isfahan province (Iran) for a period of 3 months (from March 2013 to May 2013). The camels (*Camelus dromedaries*) were apparently healthy at the time of slaughter and none were previously immunized against *Brucella* spp. Also, these animals were imported from Pakistan at the end of 2012 and at the beginning of 2013 (the camels were not serologically tested for brucellosis).

All samples were collected under sterile hygienic conditions. From each animal, 10 ml of whole blood (with anticoagulant) was aseptically taken and immediately divided into 10  $\mu$ l aliquots in tubes containing EDTA and used for PCR. After slaughtering the animals, lymphoid tissue was sampled from the subscapular lymph nodes and immediately placed in sterile containers. 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 ( $-20^{\circ}\text{C}$ ) until analysis.

### ***DNA extraction from blood and lymph tissue samples***

DNA from the 246 blood and lymph tissue samples was extracted using the CinnaGen DNA extraction kit<sup>TM</sup> (Cinnagen, Tehran, Iran) according to the instructions of the manufacturer. Total DNA was measured at 260 nm according to the method described by Sambrook and Russell [9].

### **DNA amplification and detection of PCR products**

The PCR reaction mixtures were placed in a Corbett Palm-cycler (Corbett Research, Australia). Genus-specific PCR primers 1 (Bru-F: 5' CTATTATCCGATTGGTGGTCTG 3' and Bru-R: 5' GGTAAAGCGTCGCCAGAAGG 3') were used to amplify a 245 bp for the detection of *Brucella* spp. [4], and primers 2 (Ba-F: 5' GACGAACGGAAITTTTCCAATCCC 3' and RR: 5' TGCCGATCACTTAAGGGCCTTCAT 3') were used to amplify a 494 bp for *Brucella abortus* [10] and primers 3 (Bm-F: 5' AAATCGCGTCCTTGCTGGTCTGA 3' and RR: 5' TGCCGATCACTTAAGGGCCTTCAT 3') to amplify a 734 bp for *Brucella melitensis* [10].

PCR was carried out in a total volume of 25 µl, using 50 mM KCl, 10 mM Tris-HCl (pH 8), 1.5 mM MgCl<sub>2</sub>, 0.2 mM each of the four deoxynucleotide triphosphate and 0.05 IU of Taq polymerase (Roche applied science, Germany), 0.4 mM of each primer, and 2 µl template DNA.

The amplification was performed in a DNA thermal cycler at a denaturation temperature of 95°C for 5 min; followed by 35 cycles at 94 °C for 45 s, 64.9 °C for 1 min, and 72°C for 1 min and one final extension at 72°C for 7 min, with a final hold at 4°C in a DNA thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany).

Distilled water instead of template DNA was routinely used as the negative control in each PCR together with the DNA samples to eliminate the effect of contamination. Positive controls with genomic DNA of *Brucella* were included in each run to detect any amplicon contamination or amplification failure.

### **Electrophoresis**

The PCR products were loaded in a 1% (w/v) agarose gel containing 1× TBE buffer (100 mM Tris-HCl (pH 8), 90 mM boric acid, and 1 mM Na<sub>2</sub>EDTA), stained with an ethidium bromide solution (0.5 µg/ml) and a DNA ladder (Fermentas Co., Germany) used to detect the molecular weight of observed bands and visualized under UV light. Also, images were obtained in UVIDoc gel documentation systems (Uvitec, UK).

### **Statistical analysis**

Data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using the Statistical Package for the Social Sciences (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA).

## **RESULTS**

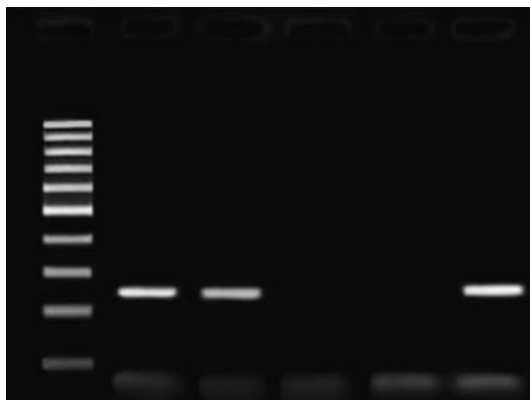
In this study, blood and lymphoid tissue samples were collected from 123 camels and all of the samples were examined using PCR technique. The number of positive *Brucella* samples obtained from slaughtered camels is shown in Table 1.

**Table 1.** Prevalence of *Brucella* spp. in camel samples

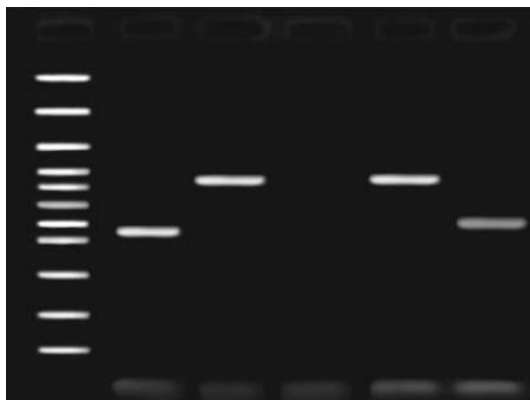
Samples	No. of animals tested	PCR <i>Brucella</i> spp.		PCR <i>B. abortus</i>		PCR <i>B. melitensis</i>		PCR positive for other <i>Brucella</i> (%)	PCR positive for both bacteria ( <i>B. abortus</i> + <i>B. melitensis</i> ) (%)
		positive (%)	negative (%)	positive (%)	negative (%)	positive (%)	negative (%)		
Blood	123	14 (11.4)	109 (86.6)	5 (4.1)	118 (95.9)	3 (2.4)	120 (97.6)	6 (4.878)	2 (1.626)
Lymph node	123	16 (13)	107 (87)	4 (3.3)	119 (96.7)	2 (1.6)	121 (98.4)	10 (8.130)	2 (1.626)
Total	246	30 (12.195)	216 (87.804)	9 (3.658)	237 (96.342)	5 (2.032)	241 (97.967)	16 (6.504)	4 (3.252)
Sex									
Male	78	7 (8.974)	71 (91.025)	3 (3.846)	75 (96.153)	1 (1.282)	77 (98.718)	3 (3.846)	1 (1.282)
	L	7 (8.974)	71 (91.025)	2 (2.564)	76 (97.435)	1 (1.282)	77 (98.718)	4 (6.410)	1 (1.282)
Female	45	7 (15.556)	38 (84.444)	2 (4.444)	43 (95.556)	2 (4.444)	43 (95.556)	3 (6.666)	1 (2.222)
	L	9 (20)	36 (80)	2 (4.444)	43 (95.556)	1 (2.222)	44 (97.778)	6 (11.111)	1 (2.222)
Total	123	14 (11.382)	109 (86.618)	5 (4.065)	118 (95.935)	3 (2.439)	120 (97.561)	6 (4.878)	2 (1.626)
	L	16 (13.008)	107 (86.992)	4 (3.252)	119 (96.748)	2 (1.626)	121 (98.374)	10 (8.130)	2 (1.626)
Age									
Adult	86	10 (11.627)	76 (88.372)	4 (4.651)	82 (95.349)	2 (2.325)	84 (97.675)	4 (4.651)	1 (1.162)
	L	10 (11.627)	76 (88.372)	3 (3.488)	83 (96.512)	1 (1.162)	85 (98.738)	6 (8.139)	1 (1.162)
Young	37	4 (10.810)	33 (89.190)	1 (2.702)	36 (97.298)	1 (2.702)	36 (97.298)	2 (5.405)	1 (2.702)
	L	6 (16.216)	31 (83.784)	1 (2.702)	36 (97.298)	1 (2.702)	36 (97.298)	4 (8.108)	1 (2.702)
Total	123	14 (11.382)	109 (86.618)	5 (4.065)	118 (95.935)	3 (2.439)	120 (97.561)	6 (4.878)	2 (1.626)
	L	16 (13.008)	107 (86.992)	4 (3.252)	119 (96.748)	2 (1.626)	121 (98.374)	10 (8.130)	2 (1.626)

Findings from this study indicated that 11.38% blood samples were positive for *Brucella* spp. and 13.01% lymph node samples were positive for *Brucella* spp. by PCR method. In this study, 5 out of 123 (4.065%) and 3 out of 123 (2.439%) camel blood samples were positive for *B. abortus* and *B. melitensis*, respectively. Also, 4 out of 123 (3.252%) and 2 out of 123 (1.626%) camel lymph node samples were positive for *B. abortus* and *B. melitensis*, respectively. Moreover, 4.88% camel blood samples and 8.13% lymph node samples were positive for other *Brucella* species.

All samples (blood and lymph samples) were tested for *Brucella* spp. where a positive band had a 245 bp (Figure 1). Also samples were tested specifically for *B. abortus* and *B. melitensis* and the positive samples indicated a band size of 494 and 734, respectively (Figure 2).



**Figure 1.** Ethidium bromide-stained agarose gel electrophoresis of PCR products (245 bp) for the detection of *Brucella* spp. in camel samples after PCR amplification. Lane 1: 100 bp DNA ladder (Fermentas, Germany); lanes 2 and 3: *Brucella* in camel (lanes 2: *Brucella* in blood sample and lane 3: *Brucella* in lymph node sample); lanes 4: negative sample; lane 5: Negative control and lane 6 is the positive control.



**Figure 2.** Ethidium bromide-stained agarose gel electrophoresis of PCR products (494 bp and 734 bp) for detection of *B. abortus* and *B. melitensis* in camel samples after PCR amplification. Lane 1: 100 bp DNA ladder (Fermentas, Germany); lanes 2 and 6: *B. abortus* in camel (lanes 2: *B. abortus* in blood sample and lane 6: *B. abortus* in lymph node sample); lanes 3 and 5: *B. melitensis* in camel (lanes 2: *B. melitensis* in blood sample and lane 6: *B. melitensis* in lymph node sample); lanes 4: Negative control.

## DISCUSSION

Camels are widespread and multipurpose animals in Iran; over 200,000 dromedary camels live in the arid and semiarid deserts of Iran [2]. However camel brucellosis has received little study, notwithstanding its importance in the transmission of *Brucella* to human beings. It is important for the reason that there is no worldwide programs for the control of camel brucellosis such as for example vaccination, testing, and slaughter of reactors.

To our data, although there are some reports on the detection of camel brucellosis in Iran and other parts of the world, this is the first time that PCR has been applied in the diagnosis of camel brucellosis (*B. abortus* and *B. melitensis*) and has obtained suitable results with good specificity. In future, an appropriate PCR technique could be used as a supplementary test for the identification and differentiation of *Brucella* in camels with the lowest risk of infection to laboratory personnel [2].

The camel plays very important socioeconomic roles and supports the survival of millions of people in the dry and semi dry zones of Africa and Asia [11]. Brucellosis is a serious zoonotic disease affecting man and all domestic animals including camels. It is considered as one of the greatest public health problems all over the world [2,11]. Brucellosis was reported in camels as early as 1931 [11], later, the disease has been reported from all camel-keeping nations [11].

Work-related acquired brucellosis is of special concern for public health due to the high risk of direct transmission from infected animals to persons being employed in animal husbandry. This exposed group includes veterinary clinicians, dairymen, slaughter men and herdsmen. Herdsmen are at the highest risk. The occupational exposure is high, especially in nations where herding of animals is still traditional and unscientific [11]. Through the development of commercial camel dairies in some nations, this disease should be considered because of its impact on human health. Regrettably, until now, there are no studies on eradication strategies or vaccination of camel brucellosis [11].

Since the discovery of brucellosis, many articles on investigations into brucellosis in camels, cattle, sheep and goats, and human beings have been published [4,12-17]. Serological evidence for *Brucella* infection in camels has been reported in Asia and Africa [18-20]. The isolation of *Brucella* spp. from internal organs (particularly lymph nodes, testes and vagina) has previously been done [3,4]. Isolation of *B. melitensis* and *B. abortus* from camels' milk has also been reported [11,21].

The prevalence of camel brucellosis from different nations may be attributed to changing husbandry and management practices, the number of affected camels, the virulence of the organisms, absence of veterinary service, presence of reactor animals in the area, lack of awareness about the disease in camels and continuous movement of infected camels into a camel herd [11]. Camels can be infected via the alimentary tract from contaminated feed or water, via the respiratory system with contaminated dust or droplets, and via the genital system from infected semen [2,11].

Malta fever caused by *B. melitensis* was detected in 30% of camel milkers and handlers on a large camel farm in Riyadh, Saudi Arabia. The abortion rate on the farm, reached 12% and *B. melitensis* biovars 1, 2 and 3 were isolated from aborted camel fetuses [11]. Consequently, there is a real need for a cooperation between public health officials and veterinary officers to decrease the circulation of human brucellosis in endemic areas [22].

A number of researchers have used different serological tests (RBT, CFT, Serum Agglutination Test (SAT), Competitive Enzyme Linked Immunosorbent Assay (cELISA), Indirect Enzyme Linked Immunosorbent Assay (iELISA), and Mercapto-ethanol test (2ME) for the finding of camel brucellosis [7,23,24]. Seroprevalence of camel brucellosis appear to follow two distinct patterns i.e. a low prevalence below 5% in nomadic or extensively kept camels and a high prevalence of 8-15% in camels kept intensively or semi intensively [3]. More-successful isolation of *Brucella* was reported from lymphoid tissues than any other organ [3].

*B. melitensis* was isolated from camels in many countries such as Iran, Libya and Saudi Arabia; *B. abortus* was isolated in Egypt, Kuwait and Sudan. It is likely that the tendency of Saudis to raise large flocks of sheep along with the camel herds contributed towards the spread of *B. melitensis* among camels [3]. Warsame et al., (2012) studied camel brucellosis and reported an overall seroprevalence of 1.5% (n=646) [19]. Teshome et al., (2003) reported camel brucellosis with a seroprevalence of 5.7% and 2.8% in Afar and Somali regions in Ethiopia [25]. The Tilahun et al., (2013) study showed a 2.43% overall seroprevalence of camel brucellosis in Eastern Ethiopia [5].

High seroprevalence of camel brucellosis has been recorded in Sudan 30.5% [26], in Darfur (Western Sudan) 23.8% [27], in Jordan 19.4% [28], and in Egypt 7.3% [29]. Zewold and Haileselassie (2012) studies on brucellosis from 768 camel serum samples indicated 11.9% positive reactors for RBPT and 7.6% for CFT [30].

Musa et al. (2008) reported a higher prevalence of brucellosis (23.8%) from camels kept concurrently with other ruminant species; they recommended that cattle were the likely source infection for the camels as small ruminants were seronegative [27]. The seroprevalence of brucellosis was three to four-fold higher among adult camels than young ones and two-fold higher in females compared to males [3]. Human infection caused by *Brucella* from camels is known to happen typically through the consumption of raw milk [11].

Control of camel brucellosis should be a must for nations where camels are raised. Vaccination of uninfected animals is conventionally considered as the most effective and economical mean of defending farm animals against brucellosis [31].

The current research showed that the prevalence of camel brucellosis was low. Even though, prevalence of camel brucellosis is low, the positive animals may serve as future foci of infection, leading to low productivity, posing a public health risk and lowering the market value of camels. The low prevalence observed in the present research might be due to the low density of the camel population kept in a widely extended



grazing land and the presence of several watering points in the river path of the valleys which reduce the concentration and close contact of camels. Moreover, the good practice of herders of timely culling non-conceiving females and removing aborted fetuses from the herds might have contributed to the situation.

These findings suggest that control and eradication programs for *Brucella* spp. infection in Iran should be taken into consideration. Our findings support the power of PCR testing for *Brucella* spp. detection in blood and lymph node samples and could be mainstreamed in routine diagnosis of brucellosis.

In conclusion, camels play a significant role in the epidemiology of brucellosis; the probability that brucellosis may spread from camels, and the lack of detailed epidemiological research of the disease in camels strongly calls for a check of the prevalence of the disease. Moreover, camel brucellosis should be included in national programs for the control and eradication of brucellosis in endemic nations. So, individuals working with these animals should be aware about the risk of camels as a source of brucellosis. Further detailed research involving different possible risk factors in camels, humans and other animals in a wider area is suggested and will allow an effective control program to be designed and help as a baseline for supplementary study.

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## **MOLEKULARNA ISPITIVANJA PREVALENCIJE *BRUCELLA ABORTUS* I *BRUCELLA MELITENSIS* U UZORCIMA KRV I LIMFNIH ČVOROVA ŽRTVOVANIH KAMILA METODOM LANČANE REAKCIJE POLIMERAZE (PCR) U IRANU**

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Bruceloza je zoonoza koja se karakteriše smanjenom sposobnošću reprodukcije i abortusima kod nekoliko vrsta životinja i ljudi. Brucelozu kod kamila izazivaju *Brucella abortus* i *Brucella melitensis*. Standardne dijagnostičke tehnike kao što su izolacija na hranljivim podlogama i serološke reakcije nisu pouzdane i imaju značajna ograničenja. Da bi se to izbeglo, radi dokazivanja bruceloze kod 123 kamila, upotrebljena je osetljiva dijagnostička molekularna metoda: PCR. Rezultati su pokazali da je 11,38% ispitanih uzoraka krvi i 13,01% uzoraka limfnih čvorova bilo pozitivno na *Brucella* spp. U studiji,

od ukupno 123 uzoraka krvi, 5 uzoraka (4,065%) je bilo pozitivno na *B. abortus*, a 3 (2,439%) na *B. melitensis*. Od ukupno 123 uzorka krvi, 4 uzorka (2,252%) su bila pozitivna na *B. abortus*, a 3 (1,626%) na *B. melitensis*. Najveća prevalencija je bila u grupi mladih životinja, a starije kamile su retko oboljevale. Veća je prevalencija ustanovljena kod ženki. Rezultati ukazuju da je PCR osetljiva metoda koja može da se koristi kao potvrdni test za dokazivanje bruceloze kod živih životinja uz minimalan rizik od infekcije laboratorijskog osoblja koje obavlja dijagnostiku. Dobijeni rezultati ukazuju da postoji neophodnost definisanja programa kontrole i iskorenjivanja bruceloze kamila. Istovremeno, rezultati ukazuju na pouzdanost PCR metode u dokazivanju *Brucella* spp bakterija, u uzorcima krvi i limfnih čvorova, što čini ovaj metod pogodnim za rutinsku dijagnostiku bruceloze.