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Identification of *Coxiella Burnetti* and *Mycobacterium* SPP through Touch-down PCR Examination in Unpasteurized Camel Milk in North-East of Iran

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ABSTRACT

Introduction: Food-borne illness cause major international health problems and reduce economic growth. A highly prevalent zoonotic disease is Q fever, found in many areas like New Zealand, Saudi Arabia, and Egypt. *Coxiella burnetii* is caused by an obligate intracellular bacterium that is considered in ruminants and ubiquitous and can survive in the environment for a long time. Early and reliable diagnosis of food borne pathogens through molecular methods like polymerase chain reaction is critical to find positive outcomes in eradication programs.

Method: In this study 100 milk samples obtained from 100 camels were examined in terms of *C. burnetii* and Mycobacterium presence through a Touch-down PCR assay.

Results: In total, there were six positive specimens of *Coxiella burnetii* in camel milk samples. No Mycobacterium was found in the samples.

Conclusions: The findings indicated that healthy camels were major sources of *C. burnetii* in North-East of Iran. There is a need for studies on risk of *Coxiella* infection in farmers, veterinarians, milk-processing and slaughterhouse workers.

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Introduction

Q fever due to *Coxiella burnetii* is a known disease caused by a ubiquitous gram-negative and resistant bacterium that can infect humans and animals including mammals (1). Q fever is a global health issue in humans with subclinical, acute and chronic forms (2). In the acute form, it appears as a self-limiting flu illness, and in chronic form, it manifests as a progressing infection (e.g., endocarditis and premature delivery in pregnant women) (3). *C. burnetti* is gram-negative resistant bacteria. It can be tolerated under pressure, heat, chemical stress, or months of hard situations. (4). many animal species are vulnerable to *C. burnetti* such as carnivores, ruminant and non- human primates (5). Sheep, goats or cattle are known as important sources of human infections (2). In these animals, *C. burnetii* infection is subclinical in most cases (6). If signs of the disease, referred to as *Coxiellosis*, manifests, reproductive complications like abortions, stillbirth, or weak newborns are observed (7). Inhalation of respiratory secretions, especially contaminated aerosols with secretions of the aborted fetus secretions are the main transmission route. During parturition in sheep and goats high load shedding of *C. burnetii* with about 10^9 bacteria per gram of placenta can be detected (6). the bacterium shed into milk, urine, and faces.

Mycobacterium tuberculosis complex is the major source of food-borne tuberculosis in humans is mostly related to the *M. Tuberculosis* complex (8). Animal tuberculosis (TB) is widely distributed with a wide range of domestic and wild reservoirs (9).

Many studies have shown that camel milk is highly identical to human milk among other animals. The milk is different from other ruminant milk with lower sugar, cholesterol,

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higher minerals (potassium, sodium, copper, zinc, iron, and magnesium), and vitamin (10). Camel milk has unique specifications compared to other ruminants and it has functional effects on humans' effects. The milk can be easily digested by people who are lactose-intolerant with potential medical effects such as anti-diabetic, anti-hypertensive, and anti-carcinogenic (10).

The proteins found in camel milk create a balance of essential amino acids. The milk has immunoglobulins that fight diseases with small size so that they can enter antigens and enhance the efficiency of the immune system (11).

This study aimed to detect *C. burnetii* and *Mycobacterium spp* in raw camel milk. The samples were collected from camels of Mashhad city using a touchdown PCR assay.

Material and Methods

Experimental Procedures

DNA Extraction from Raw Milk

To remove layers and cream, the milk was centrifuged and then DNA was extracted using Berri et al (2000) method (12), with some modifications. 1ml volume of the camel milk samples was transferred to a 1 ml for three times centrifuging at 15000 rpm for 5min. every time the supernatant was removed and refilled by normal saline. The isolation of DNA was performed with the help of a genomic DNA extract kit (Dena Zist, Iran) based on the producers' instructions. The quantity of the extracted DNA was measured through spectrophotometry (Nano Drop™ 2000/2000c spectrophotometer; Thermo Scientific, USA). The concentration of DNA and purity were measured under UV absorbance at 260 nm and by the ratio of absorbance at 260 and 280 nm, respectively. (13).

DNA amplification (trans-PCR)

A pair of primers were used in the PCR assay to measure the IS*1111* gene transposase elements *C. burnetii* genome. Trans-1 (5'-TAT GTA TCC ACC GTA GCCAGT C-3') and trans-2 (5'-CCC AAC ACC TCC TTATTC-3') primers were utilized based on the available data (14) which amplified a 687-bp fragment of the target sequence. The PCR examination was carried out as mentioned by (15). The PCR mixture (25µL) contained 2.5µL of 10×PCR buffer (100 mM Tris-HCl buffer, pH 8.3, 500 mM KCl, 15 mM MgCl2, and 0.01% gelatin, 200 µM deoxynucleoside triphosphate mix, 2µM of each primer, 0.3 U of Taq DNA polymerase, 3µL of template DNA, and deionized water. To perform the amplification, a thermal cycler (TECHNE TC- 5 UK) and the cycling program were as followed: DNA denaturation (95°C; 2min), five cycles (94°C for the 30s, 66 to 61°C; the temperature decrement by 1°C in consecutive steps) for 1 min, and 72°C for 1 min. After the initial cycling program, another 35 cycles (of 94°C for 30 s, 61°C for 30 s, and 72°C; 1min) and then a final extension step of 10 min at 72°C (14) were performed. Following electrophoresis in the agarose gel and green viewer staining (Sinaclone), they were examined using UV illumination. Following the first positive PCR product, which was detected as *C. burnetii* by sequence analysis, the product was utilized as a positive control and deionized distilled water was the USA as negative control.

To identify the genus Mycobacterium, the um, Hsp65 gen, us is used, and to identify the specific species of *Mycobacterium tuberculosis*, Primer 6110 (IS6110) Insertion sequence is used (16,17) Amplification was performed in a 25 µL reaction volume with 12.5 µL Taq DNA Polymerase Master Mix RED, 1 µM of each of the primers and 4 µL of template DNA and 6.5 µL Sterile distilled water. *Mycobacterium tuberculosis* H37Rv was considered as positive control while the negative control. The PCR outcome was examined using 1.5% agarose gel electrophoresis, after which the gel was marked using green viewer. This standard marker was supplied by Pars Toups, Iran.

IS1111 gene *(C. burnetii*)

Table1. Primer sequences for detection of C. burnetii IS1111 gene by nested PCR

Primer name	Primer Sequence	Amplicon size
Trans 1	5'-TAT GTA TCC ACC GTA GCCAGT C-3'	**687bp**
Trans 2	5'-CCC AAC ACC TCC TTATTC-3'	

Hsp65 (Mycobacterium)

Amplification was carried out in a thermal cycler for 35 cycles (95C for 5min as the early initial denaturation; 95C and 30s, 62C and 30s, 72 C and 45s, and final extension at 72 C and 10 min.

Table2. Sequence and PCR primer of Mycobacterium IS6110 (Tuberculosis) Amplicon size (441 bp)

Primer name	Primer Sequence	Amplicon size
TB11F	5'-ACC AAC GAT GGT GTG TCC AT-3	**441bp**
TB12R	5'-CTT GTC GAA CCG CAT ACC CT-3	

Amplification was carried out in a thermal cycler with 35 cycles (95C for 5 min as starting, cycling of 95 C and 30 s, 68 C and 30 s, 72 C and 45 s, and 72 C and 10 min).

Table3. Sequence and PCR primer of Mycobacterium IS6110 (Tuberculosis) Amplicon size (123 bp)

Primer name	Primer Sequence	Amplicon size
INS1F	5'- CCTGCGAGCGTAGGCGTCGG -3'	**123bp**
INS2R	5'- CTCGTCCAGCGCCGCTTCGG -3	

Results

Using touch-down PCR assay to target the IS*1111* gene of the organism through Trans-1 and trans-2 primers, 6 positive *C. burnetii* out of 100 samples (6%), (in the case of positive control, the 687 base pairs of the amplified gene fragment were sequenced successfully out of the first PCR-positive sample. We compared library gene sequences of *C. burnetti* with our samples, and there was no difference between deduced amino acid and nucleotide (Figure 1).

To identify the genus Mycobacterium, all 100 DNA samples were extracted by PCR with Hsp65 primer, none of which showed a band of 441 bp. This result indicates that none of the milk samples were infected with Mycobacterium. There was no need for PCR to identify the species.

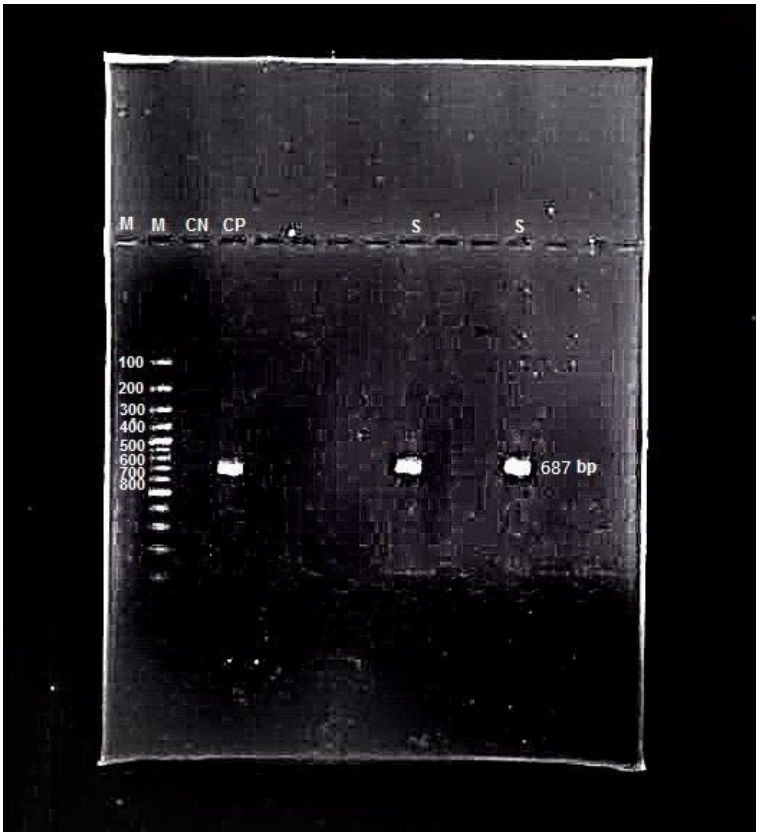

Figure 1. Electrophoresis of the amplification products of the touchdown polymerase chain reaction assay.
Detection of *Coxiella burnetii* in camel milk using touch-down PCR assay, amplifying a 687 bp
Segment of the IS1111 gene: CN= Control Negative /CP= Control positive/ Line 8 and 11 are positive samples

Discussion

Coxiella burnetii is classically a strict intracellular short (0.3 to 1.0 µm), gram-negative, and pleomorphic rod organism. It causes infection in humans through contact with sick animals. *Coxiella burnetii* resist pressure, heat, chemical stress, and even several months under stressful conditions (4). The organism is also very infectious and even one *Coxiella burnetii* can cause infection, in experimental conditions (18)

C. burnetii can be shed from feces, urine, and milk of infected cases with a high level of concentration during parturition. It is mainly spread by birth products and the placenta in particular. When in chronic condition, the mammary glands and uterus are the main sites of infection of *C. burnetii* (19). The major part of transmission in the case of human hosts is dust containing birth fluid, urine, placenta, feces, or respiratory aerosols. Several studies have reported *C. burnetii* DNA prevalence in the blood of ruminants; so that bacterial DNA was found in two goats (5.26%) and 13 camels (15.85%) in Saudi Arabia. Still, the sheep and cattle under study showed no positive case (20). In Zambia, *C. burnetii* rates in goats and cattle were 7.55% and 7.77% (21). Another research work examined 13 blood samples in China and found that all the samples were negative (22). In Korea, 57 samples out blood samples from goats had positive amplification for *C. burnetii* DNA (23). The inconsistency of findings about the prevalence of the infection in these studies can be because of changes in study methods, types of herds, different sampling methods, and environmental conditions. The epidemiology of Q-fever in Iran is unclear. The first study was done by Rahim et al., in 2010. Using nested PCR assay, Rahimi examined 376 bulk milk samples supplied by 79 dairy ovine, bovines, and caprine herds in terms *of C. burnetii*.

In another study, (24) examined *C. burnetii* in milk samples of ruminant animals in Iran. They used the PCR technique for the identification of *C. burnetii.* In total, 9 (1.8%) milk samples were *C. burnetii* positive (6 goat milk and 3 sheep milk samples). The positive milk was collected from two villages (Daraei and Ghalebi) near the Khorramabad region in winter. Still, the collected samples of Nourabad were all negative.

In the present study, the PCR technique was used to target the repetitive transposon-like region (Trans-PCR) was used to investigate *C. burnetii* in camel milk samples in Iran and six out of 100 samples (6%) were positive. The method's efficiency in terms of detecting Coxiella in milk has been supported by studies. The method can detect *C. burnetii*-cell in 1 ml of milk (25). In addition, trans-PCR is highly sensitive with considerable specificity (25-28). No *Mycobacterium spp.* was found in all the milk samples in this work.

Conclusion

This study found that detects *C. burnetii* and *Mycobacterium spp.* in camel milk by touch-down PCR assay. This result showed that camel milk plays an important reservoir of *C. burnetii*. *C. burnetii* prevalence in Iranian camels was about 10.76 %; this suggests that camels can have a notable role in the epidemiological study the human population in Iran.

So it is recommended that raw camel milk should be consumed with precaution and be commercially pasteurized at 74 C for public consumption.

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Conflict of Interest

There is no conflict of interest to declare.

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