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High prevalence and risk factors of *Coxiella burnetii* in milk of dairy animals with a history of abortion in Iran

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ABSTRACT

Coxiella burnetii is causative agent of Q fever, which is a public health problem in most countries. The aim of this study was to study the prevalence rate of *C. burnetii* in raw milk of dairy animals in Iran with previous history of abortion. In this survey, milk samples were collected from different dairy animals with history of abortion from Qom province (center of Iran). Samples were tested by Nested PCR and Real-time PCR for detection of IS1111 gene of *C. burnetii*. In total, 34.92% (44 of 126) milk samples were positive for *C. burnetii*. Prevalence of *C. burnetii* in cattle, sheep and goat milk was 33.33%, 35.71% and 35.71%, respectively. Age was a significant risk factor for shedding of *C. burnetii* in cattle ($P = 0.02$) and goat ($P = 0.05$). Shedding of *C. burnetii* was high prevalence in milk of dairy animals with history of abortion in Iran. The high prevalence of this bacterium in milk (especially in animals with history of abortion) indicates that Excreted by milk as a potential source to spread of infection in the environment.

1. Introduction

Coxiella burnetii is an obligate intracellular bacterium and the etiological agent of Q fever infection in human and animals. Center for Disease Control and Prevention (CDC) classifies *C. burnetii* as a Category B pathogens with potential use for biological weapons, because this bacterium can be rapidly spread, walking long distances, and is easily aerosolized [1]. Furthermore, the infectious dose of this bacterium is very low and 1–10 organisms can cause disease in human by inhalation of infected aerosols [2]. *C. burnetii* has a spore-like structure in the environments and is thus able to persist in harsh environmental conditions with extensive physical and chemical stresses [3].

Q fever is a zoonotic disease and has been reported in all countries around the world except in New Zealand. *C. burnetii* can infect different host species (domestic and wild mammals, marine mammals, reptiles, arthropods, and birds). Domestic ruminants (cattle, sheep, and goats) are considered as the main reservoirs for this bacterium [1]. Q fever infections in animals are mostly asymptomatic, but abortion, stillbirth, infertility, endometritis and metritis has been reported in some infected livestock [4]. Infected animals shed *C. burnetii* mainly thought urine, milk, feces and especially birth or abortion products. The shedding of this bacterium in vaginal mucus, feces, and milk by infected animals can persist for several months [1,5]. Inhalation of infected aerosols of *C. burnetii* is the main route of transmission to humans. Direct contact, consumption of unpasteurized dairy products, tick bites, and human-to-human transmission are less common, yet important routes of infection transmission to humans [6]. At-risk individuals of this disease include livestock breeders, shepherds, veterinarians, butchers, abattoir workers, farmers, dairy workers, laboratory staff, and people in contact with domestic animals, especially during livestock parturition [1,6].

In humans, clinical manifestations of Q fever include acute or chronic, Q fever fatigue syndrome and abortion. Acute Q fever is the primary form of infection by *C. burnetii*, and more than half of the patients are asymptomatic. Acute Q fever is usually presented as a non-specific febrile and self-limited illness, pneumonia, or hepatitis [1,6]. About 1–5% of the acute infection cases go on to develop chronic Q fever, which may manifest months or years after an initial infection. Endocarditis, vascular infection, prosthetic joint arthritis, osteoarticular infection and lymphadenitis are major clinical manifestations of chronic Q fever. In the untreated endocarditis patients with appropriate antibiotics, death is inevitable [1].

In Iran, Q fever is an endemic disease. Based on a recent systematic

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review conducted in Iran, Q fever had high seroprevalence among human and domestic animals population [7]. *C. burnetii* has been identified by molecular method from milk and dairy products, fetus and ticks. Recently acute and chronic Q fever cases were also reported in Iran [8–10]. Despite all this, Q fever is a neglected disease in Iran, this could be due to the fact that clinical physicians and healthcare workers do not receive adequate training to be able to identify the disease and Q fever is not a reportable disease by health system in Iran.

The aim of this study was to investigate the prevalence of *C. burnetii* in raw milk of dairy animals in Iran with previous history of abortion. Although some recent studies have been conducted on the prevalence of *C. burnetii* among livestock in Iran, information about *C. burnetii* presence in milk is incomplete in large parts of Iran. Our work is an effort to attract more attention to the Q fever disease and *C. burnetii* by the national healthcare system and veterinary organization in Iran.

2. Material and methods

2.1. Study Area

This cross-sectional study was carried out in Qom province in 2017. This province is located approximately in the central of Iran and neighbors Tehran province from north, Semnan province from east, Isfahan province from south, and Markazi province from west and southwest. This province covers an area of 11,240 Km^2. The climate of Qom province is varying semi-desert to desert. Annual rainfall averages 161 mm and this province has a human population of 1,292,283, of which 95.18% reside in urban areas. Qom province has more than 142,000 of cattle, 180,000 goats and 720,000 sheep.

2.2. Sampling

This study was approved by the Ethics Committee of Biomedical Research of Tarbiat Modarres University (Ethic Code: IR.TMU.REC.1395.510). Sampling was conducted from June to July 2017. Farms that had a history of abortion were identified on the basis of information recorded (during the last three years) in the Veterinary Office of Qom province. The dairy farms (goat, sheep and cattle) were randomly selected. Information for each herd and dairy animal was recorded. In each herd, 50 mL raw milk was collected from each diary livestock that had abortion history during the last three years. Milk samples were immediately transported to the laboratory under the cold chain (4 to 8 °C).

2.3. Milk processing and DNA extraction

After transferring of samples to the laboratory, milk samples were centrifuging at 4500 rpm for 15 min in 50 mL falcon tube and removed the cream layer. The supernatant was discarded and the sediment was re-suspended using 50 mL sterile phosphate buffered saline (PBS). Samples were then centrifuged at 4000 rpm for 10 min. The supernatant was discarded and the precipitate was dissolved in 20 mL PBS solution and centrifuged for 10 min at 4000 rpm. The final precipitate was dissolved in 1 mL PBS and stored at -20 °C until extraction of DNA.

For DNA extraction, 200 µL of the final sediment solution in the previous step was used. Genomic DNA was isolated using the Roche High Pure PCR Template Preparation Kit (Roche, Germany), according to the manufacturer's instruction.

2.4. Molecular detection

All samples were tested by nested PCR and real-time PCR for detection of IS1111 gene of *C. burnetii* (Table 1). Nested PCR method was performed via two runs of PCR using two sets of primers including Trans1 and Trans2 for first amplification followed by 261 F and 463R for second amplification reaction [9]. The products of first PCR were separately used as DNA template in a second round of PCR. Each PCR reaction contained 5 µL of DNA, 12.5 µL Taq DNA Polymerase Master Mix RED (Ampliqon, Denmark), and 10 pmol/µL from each primer in a final volume of 25 µL. PCR was performed in a thermal cycler (Bioneer, South Korea). The first amplification of PCR was 95 °C for 2 min, followed by five cycles at 94 °C for 30 s, 66 to 61 °C (touchdown assay) for 1 min and 72 °C for 1 min. These cycles were followed by 35 cycles consisting of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min, then a final extension step of 10 min at 72 °C. In the second amplification, the cycling conditions included an initial denaturation of DNA at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 50° for 45 s, 72 °C for 1 min, then a final extension step of 10 min at 72 °C. The amplicons were electrophoresed on 1.5% agarose gel and visualized under UV.

Real-time PCR was performed using specific primers and probe sequences targeting IS1111 gene (Table 1). Real-time PCR reactions were performed using the following reaction mixture: 10 µL of 2x RealQ Plus Master Mix for Probe (Ampliqon, Denmark), 900 nM forward primer, 900 nM reverse primer, 200 nM probe and 4 µL of DNA template. Real-time performed on the Corbett 6000 Rotor-Gene system (Corbett, Victoria, Australia), with a final volume of 20 µL for each reaction. The PCR amplification program were 10 min at 95 °C, followed by 45 cycles of 15 s at 94 °C and 60 s at 60 °C [11].

2.5. Data analysis

Data were analyzed using SPSS statistical software, v. 24 (SPSS Inc., Chicago, IL). The regression logistic test was used to compare the variables during analysis. P-values less than 0.05 were considered statistically significant.

3. Results

In this study, 126 individual raw milk samples were collected from animal with abortion history. Results of nested PCR were completely consistent with Real-time PCR results for detection of *C. burnetii* DNA based on the IS1111 amplification. In total, 44 (34.92%) samples were positive (Table 2).

C. burnetii was detected in 33.33% (95% confidence interval [CI]:21.01–48.45) of cattle milk samples. All six sampled cattle herds were positive. Herd level prevalence was 34%. The highest and lowest prevalence in herd level was 75.34% and 14.29%, respectively. Regression logistic analysis showed that age was a significant risk factors for shedding of *C. burnetii* in cattle milk ($P = 0.02$). Moreover, chance for a positive result increased 1.67 times (95% CI: 1.10–2.53) for an increase in each year age. Time of abortion was a weak risk factor for cattle ($P = 0.06$) (Table 3).

Prevalence of *C. burnetii* in samples of sheep milk was 35.71% (%95CI: 20.71–54.17). Herd level prevalence was 40.21%. No positive cases were found in the three herds and all milk samples were positive in two flocks. Furthermore, no significant relationship was found between risk factors and shedding of *C. burnetii* (Table 3).

C. burnetii was detected 35.71% (%95CI: 24.46–48.81) in goat's milk samples. Herd level prevalence was 42.36% in goat. No positive cases were found in the three flocks, and all milk samples were positive in two flocks. Regression logistic analysis showed that age was significant risk factors for shedding of *C. burnetii* in goat's milk (($P = 0.05$), OR = 0.61 (95% CI: 0.38-0.99)). Others risk factors were not significant (Table 3).

4. Discussion

In this study, we investigated raw milk samples of animal with abortion history to detection of *C. burnetii* by nested PCR and real-time PCR in central Iran. The prevalence of *C. burnetii* was very high (34.92%) in raw milk samples. The high prevalence of this bacterium in milk (especially in animals with history of abortion) indicates that

Table 1
Primer sequences for diagnosis of *C. burnetii* IS1111 gene by Nested PCR and Real-time PCR.

Protocol	Primer	Sequence (5→3)	Amplicon size (bp)
Trans-PCR	Trans1	TATGTATCCACCGTAGCCAGTC	687
	Trans2	CCCAACAACACCTCCTTATTC	
Nested PCR	261F	GAGCGAACCATTGGTATCG	203
	463R	CTTTAACAGCGCTTGAACGT	
Real-Time PCR	tmQ-koorts4-fw	AAAACGGATAAAAAGAGTCTGTGGTT	70
	tmQ-koorts4-rv	CCACACAAGCGCGATTCAT	
	tmQ-koorts4-fam-tamra	6-FAM-AAAGCACTCATTGAGCGCCGCG-TAMRA	

Table 2
Prevalence of *C. burnetii* in milk of dairy animal with abortion history in the Qom province.

	Number of sampled flock	Number of samples	Number of Positive samples	Rate of positive samples (%95CI)
Cattle	6	42	14	33.33 (21.01–48.45)
Sheep	9	28	10	35.71 (20.71–54.17)
Goat	12	56	20	35.71 (24.46–48.81)
Total	27	126	44	34.92 (27.16–43.58)

Excreted by milk as a potential source to spread of infection in the environment. The shedding of *C. burnetii* by ruminants is an important public health threat [12]. Some studies have reported higher seroprevalence and clinical disease in patients consuming raw milk [13]. Unfortunately, the tradition of consuming dairy products made from unpasteurized milk, especially amongst those living in rural areas and remote regions, increases the risk of diseases caused by milk-borne pathogens [14]. Contamination of raw milk can further lead to generation of contaminated aerosols during various stages of milk manipulation, including milking of livestock and handling of milk at the farms and dairy factories. Therefore, it is necessary to take measures to raise awareness regarding preventive methods such as pasteurization of milk and use of personal protective equipment and appropriate containment when dealing with livestock.

In our results, the shedding of *C. burnetii* in individual milk was 33.33% in cattle dairy. These livestock did not have any clinical signs and only had a history of abortion. In a previous study from 2011, 14% of cattle bulk milk were positive for *C. burnetii* in Qom province [15]. This difference in prevalence of *C. burnetii* can be the result of these factors: (I) use of multicopy gene (IS 1111) for detection in our study versus use of single-copy gene in previous study, (II) sampling from cattle with abortion history in our study versus random sampling in previous study, (III) individual milk sampling in our study versus bulk tank milk sampling in previous study, and (IV) increase the prevalence of *C. burnetii* over time, because there is no control and prevention measures of Q fever in Iran. Furthermore, wide range of *C. burnetii* frequency in cow milk was reported from different provinces in Iran: 5% in Khorasan-Razavi [16], 8.6% in Isfahan [17], 12% in Tehran [18], 14.9% in Zanjan [19], 17.1% in Fars [20]. Prevalence of *C. burnetii* in milk from dairy cattle was different in others countries: 4.7% in Switzerland [21], 18.8% in the Netherlands [22], 22% in Egypt [23], and 57.1% in USA [24]. Shedding via milk is the most common route of spreading *C. burnetii* in the environment by infected dairy cattle. The shedding of *C. burnetii* in milk by cattle may persist for a time longer than 1 year and may be continuous or intermittent [5,14]. In our study, all sampled cattle herds had at least one positive milk sample. Also, age of dairy cattle was a significant risk factor for shedding of *C. burnetii* to milk. These factors should be considered in the Q fever control programs.

Infected sheep excrete *C. burnetii* in their milk, feces, vaginal discharge, and parturition and abortion products. In sheep experimentally infected with *C. burnetii*, these animals shed the bacteria for two parturitions following initial abortion [25]. Based on a recent study, seroprevalence of Q fever in sheep has been estimated at 24.7% in the Iran [7]. Also, sheep with a history of abortion had higher seroprevalence compared with sheep with no history of abortion [26]. In the present study, all ewes had a history of abortion and 35.71% of ewes shed *C. burnetii* in their milk. Also, high prevalence of *C. burnetii* in sheep milk was reported from Iran and others countries: 3.3% and 34.8% in Iran [19,27], 4% in Turkey [28], 4% in Hungry [29], 19% in France [30] and 22% in Spain [31]. Although the main route of shedding of this bacterium is through feces in sheep but result of this study showed that shedding of *C. burnetii* in milk was markedly high among sheep with a history of abortion.

In goats, clinical manifestation of Q fever included pneumonia, abortion with stillbirth and delivery of weak yeanling. Excretion in milk is a major route for shedding of *C. burnetii* in goats and shedding of this bacterium can persist for a long period, especially in aborted goats. Goats having gone through abortion can excrete *C. burnetii* in milk at two successive lactation periods [32]. In the present study, *C. burnetii* was detected in 35.71% of milk samples from goats with abortion history. In southeast of Iran, 16.1% of goat milk samples were positive for *C. burnetii* [33]. Prevalence of this bacterium in goat milk was reported 2.9% in Gambia [34], 4% in Turkey [28], 6.3–12.1% in Belgium [35], 14.3% in USA [24] and 16% in France [30]. In France, shedding of *C. burnetii* into milk in aborted goats were more than goats that delivered normally [36]. In our study, age was a significant risk factor for shedding of *C. burnetii* in goat's milk and younger dairy goats were shed more than older goats.

In Iran and similar other countries, milk and dairy products made from goat and sheep milk are unpasteurized. Also, most traditional cattle farms offer milk and dairy products as unpasteurized for consumption. In our sampled farms, milk and dairy products of goat and sheep were used unpasteurized. Based on available information, the risk of Q fever infection may be not negligible by consuming infected unpasteurized milk and raw milk dairy products to *C. burnetii* [14].

Table 3
Relationship between positivity for C. burnetii in milk and risk factors.

	Sheep		Goat		Cow	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Age	0.74 (0.36–1.51)	0.41	0.61 (0.38–0.99)	0.05	1.67 (1.10–2.53)	0.02
Number of Parturition	0.78 (0.17–3.54)	0.78	1.47 (0.67–3.24)	0.34	0.81 (0.26–2.47)	0.71
Time of Abortion	0.63 (0.30–1.36)	0.24	0.74 (0.46–1.19)	0.74	1.58 (0.99–2.52)	0.06

Our results demonstrate that the prevalence of *C. burnetii* was very high and considerable in milk samples of dairy animals (goat, sheep and cattle) with history of abortion in Iran. We also showed that large scale shedding of *C. burnetii* in herd level, which may reflect the widespread distribution of this pathogen in dairy animal with history of abortion. The high prevalence of this bacterium in milk (especially in animals with history of abortion) indicates that Excreted by milk as a potential source to spread of infection in the environment.

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