# Detection of *Coxeilla brunetii* in bulk tank milk samples from dairy bovine farms using nested-PCR in Qom, Iran, 2011

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**Abstract**:

**BACKGROUND:** Q fever is a zoonotic disease caused by *Coxiella burnetii,* a species of bacteria that is distributed worldwide. In cattle, *Coxiella burnetii* infections are generally asymptomatic but can also be associated with reproductive disorders. **OBJECTIVES:** The aim of this study was to achieve molecular detection of *Coxiella burnetii* in dairy bovine milk farms using Nested PCR in Qom province, Iran. **METHODS:** From January to February 2011 (winter) and July to September 2011(summer) a total of 100 bovine bulk milk samples were equally collected from five areas of Qom. The nested PCR assay used to screen for *C. burnetii* was designed from the nucleotide sequence of the com1 gene encodin a 27-kD outer membrane protein (OMP). **RESULTS:** In this study, 14% (14 of 100) of bulk milk were positive. **CONCLUSIONS:** These results support the hypothesis of high prevalence and endemic pattern of Q fever in Qom province of Iran.

## Introduction

*Coxiella burnetii,* (a small obligate intracellular gram-negative, a dense, small and highly resistant spore-like form bacterium with a worldwide distribution) is the causative agent of acute and chronic Q fever (Maurin and Raoult, 1999). The name "Q fever" (Query Fever) was first coined in 1937 by Derrick (Derrick, 1964 ). *C. burnetii* is classified in the family of Rickettsiaceae; it belongs to the subgroup of Proteobacteria based on 16S-rRNA sequence analysis (Maurin & Raoult, 1999). *C. burnetii* is resistant to heat and drying and can survive in the environment for months. It is also highly infectious by the aerosol route. Its use would not be generating mass fatalities, but would act as an incapacitating agent (Madariaga et al., 2003). The most commonly identified sources of human infection are farm animals, such as cattle, goats, and sheep. They shed highly stable bacteria in urine, feces, milk and through placental and birth fluids. Infection via inhalation of aerosolized organisms or ingestion of raw milk or fresh dairy products has been reported in humans and animals (Fishbein & Raoult, 1992). Also, *C. burnetii* can infect even non-mammalian species including reptiles, fish, and birds (Cutler et al., 2007). Ticks are considered to be the natural primary reservoirs of *C. burnetii* responsible for the spread of the infection in wild animals and for transmission to domestic animals (Norlander, 2000). In animals, the main clinical manifestation is abortion in pregnant cattle, sheep, goats, cats and other animals (Woldehiwet 2004). *C. burnetii* is most often transmitted to humans by inhalation of an aerosol that has been contaminated with parturient products, urine, and feces of the infected animals (Tissot-Dupont & Raoult, 2008). Human infection with *C. burnetii* can be subclinical, acute, or chronic (Sawyer et al., 1987) and about 60% of infections are

asymptomatic but give rise to seroconversion. Main clinical presentations of acute Q fever are febrile pneumonia and hepatitis (Arricau-Bouvery & Rodolakis, 2005). Q fever has been described in every country with the exception of the Antarctica and possibly New Zealand (Norlander, 2000). Since there are no specific signs of Q fever, suitable laboratory tests are required for accurate diagnosis. Because *C. burnetti* does not grow on standard laboratory bacterio-logical media and its isolation takes a long time and is difficult and hazardous to perform; it requires con-fined level 3 laboratories (L3). The diagnosis of Q fever remains difficult, and epidemio-logical studies are often based only on serological investigations, including immuno-fluorescence, complement fix-ation, and enzyme-linked immuno-sorbent assay (ELISA) (Fournier et al. 1998; Raoult et al. 1990; Scola, 2002) . Polymerase chain reaction (PCR) assays provide a valuable new approach that is sensitive, easy to perform, and safe for laboratory personnel. PCR holds the promise of timely di-agnosis, since it should be positive before antibodies are detectable (Berri et al., 2003). These assays use conventional PCR, nested PCR, or real-time PCR conditions with Light-Cycler, SYBR Green, or TaqMan chemistry (Angelakis & Raoult 2010; Klee et al. 2006). The epidemiology of Q-fever in Iran is essentially unknown (Rahimi et al., 2009a), but some reports have indicated that Q-fever was endemic in Iran. The first data about epidemiology of Q-fever in Iran is related to Giroud, and Yassemi, H in 1952 (Giroud 1952). The main aim of this study was to assess the presence of *C. burnetii*  in Bulk milk samples of dairy bovine in Qom Province through Nested PCR.

## Materials and Methods

**Study Area:** Qom Province is one of the 31 provinces of Iran with 11,237 km², covering 0.89% of the total area in Iran. It is in the north of the country, and its provincial capital is the city of Qom. It was formed from part of Tehran Province in 1995. The province contains one city, four counties, nine rural districts, and 256 villages. Qom is among the major cow, sheep, and goat raising provinces in Iran. The economy is mainly based on agriculture and livestock husbandry, and so a large proportion of the population

comes into close contact with livestock (Figura 1). We divided  Qom province to five areas for sampling namely, Ghanavat, Pajian, Hajiabad, Ghomroud, Dastjerd .

**Sample Collection:** From January to February 2011 (Winter) and July to September 2011(Summer), a total of 100 bovine bulk milk samples were equally collected from five areas. *Coxiella burnetii*  infection status of the herds was unknown prior to this study, and there was no established surveillance or manage-ment targeting *C. burnetii* control. No vaccination for *C. burnetii* was performed on these farms. The animals whose milk samples collected for this study were clinically healthy and the milk samples showed physical (color, pH, and density) consistency. The samples were immediately trans-ported to the laboratory in a cooler with ice packs and were processed within an hour of collection and held frozen at -20$^{o}$C for a maximum of two weeks for processing.

**DNA Extraction:** DNA was extracted by using a 1 ml aliquot of milk which was centrifuged at $6,000 \times$ g for 10 min. The clear whey portion was suctioned out with a transfer pipette and discarded. The remaining milk solids and butterfat were used for DNA extraction using DNA extraction kit through CinaPure (Cinnagen Co., Iran) according to the manufacture's instruction. DNA was eluted in 50 µL of sterile, double-distilled, deionized water in all cases.

**Nested PCR detection of** *Coxiella burnetii***:** The nested PCR assay used to screen for *C. burnetii*  was designed from the nucleotide sequence of the com1 gene encoding a 27-kD outer membrane protein (OMP). All primers were obtained from a com-mercial source (Cinna Gen, Iran). All sequences of Primers are available in Table 1. The PCR was carried out in a 50 µL reaction volume consisting of 5 µL 10x PCR buffer, 1 µL 10 mM dNTPs, 1.25 µL of each primer (10 pmol/µL), 0.25 µL Taq DNA polymerase (5U/µL), 1.5 µL 50 mM MgCl2, 33.75 µL of dH2O, and 6 µL DNA dilution Both in PCR and nested PCR. Before the temperature cycle, denaturation was carried out at 95ºC for 5 minutes and extension at 72ºC for 10 minutes. In the first reaction, 35 cycles were performed in three phases: denaturation at 95ºC for 60seconds, hybridization at 65ºC for 45 seconds, and extension at 72ºC for 45 seconds. In nested PCR,

35 cycles were employed: denaturation at 95ºC for 60 seconds, hybridization at 69º C for 60 seconds and extension at 72ºC for 45 seconds.

## Results

In the present study, 14% (14 of 100) of bulk milk was positive in molecular survey. The highest and lowest detection have been found in Dastjerd (6/20) and Ghomroud (0/20) areas respectively. We have the most detection rate in summer, Qom Province (9/100) (Data were shown in detail in Table 2).

## Discussion

As ruminants are the main reservoir, decreasing the exposure of humans and animals to shedder ruminants is key to limiting the spread of the infection (Taurel et al. 2011). One of the main aims of epidemiology is to provide accurate information on the status of pathogens in populations. The economic costs involved; however, there are key considerations when designing the sampling methodology. Pooling individual samples may provide a valuable alternative for reducing the associated costs. In dairy livestock, this can be done by using bulk-tank milk (BTM) since this is a good, easy to collect, and representative sample of animals under milking (Ruiz-Fons et al. 2011; van den Brom et al. 2012). *Coxiella burnetii* was detected in 9 out of 21 (42.9%) samples tested by polymerase chain reaction in commercially available raw milk from the United States (Loftis et al. 2010). Prevalence of *Coxiella burnetii* infection in Dutch dairy herds, based on testing bulk tank milk and individual samples by PCR, showed that bacterial DNA was detected in the milk of 254 of 2925 (8.7 percent) lactating cows (Muskens et al. 2011). In a study carried out in Switzerland, all 81 ovine and 39 caprine bulk milk samples were negative for *C. burnetii*, using a nested PCR assay. In another study conducted in Turkey, 3.5% of single milk samples from 400 sheep of 22 flocks were positive for *C. burnetii* by a PCR assay (Ongor et al., 2004).

A serologic survey of Q fever in domestic animals in Iran was done by Khalili et al. The results showed that 35.5% of all sera were positive. Goats had a significantly higher average seroprevalence (65.78%)



Figure 1. Locations of collecting the milk samples to characterize the situation of Coxceilla brunette infection in Qom province, Iran.
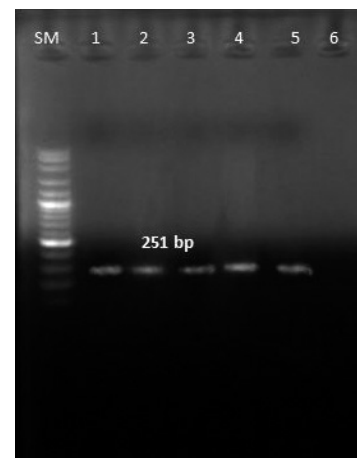


Figure 2. Agarose gel electrophoresis of *Coxiella burnetii* nested PCR products amplified from total DNA of bulk tank milk samples. SM, 1000 bp DNA ladder lane 1, positive control; lane 6, negative control; lanes 25, *Coxiella burnetii* positive milk samples from dairy bovine herds.

than cattle (10.75%) (Khalili and Sakhaee 2009). The updated results of serological study on dairy cattle farms based on bulk tank milk of large commercial dairy herds analysis in south of Iran showed that prevalence of positive, negative, and intermediate herds were 45.4%, 43.2% and 11.4%, respectively. It was the first molecular survey of C.Burnetti in Qom province. Recently, Iranian researchers conducted a study to determine the prevalence rate of *C. burnetii* in bulk milk samples in Isfahan province by Nested PCR. In total, 8 of 247 (3.2%) bovine milk samples were positive; the positive samples originated from 6 of 90 (6.7%) dairy herds. Eight of 140 (5.7%) ovine bulk milk samples from 42 sheep breeding farms and 5 of 110 (4.5%) caprine bulk milk samples from 32

Table1. Primers that used in Nested PCR detection of Coxiella burnetii.

| Primer Name | Sequences | Length |
|---|---|---|
| CF1 _ CB _ com | GTCAAAGACATACAAA GCATCGTCCACC | 540 bp |
| CR2 _CB_com | CGATTGAGCTAATTGGA AGTTATCACGC | |
| CF3 _ CB _ com | CGACCCTGCATCACCAG TGGC | 251 bp |
| CR4_CB_com | CACTGAGCAGCGCGTC GTGG | |

Table 2. Number o f Coxiella burnetii PCR positive in bulk tank milk samples of five areas of Qom Province, (winter and Summer2011).

| | PCR Positive | | |
|---|---|---|---|
| | January to February 2011 (Winter) | July to September 2011(Summer) | Total |
| Ghanavat | 2/10 | 2/10 | 4/20 |
| Pajian | 0/10 | 2/10 | 2/20 |
| Hajiabad | 0/10 | 2/10 | 2/20 |
| Ghomroud | 0/10 | 0/10 | 0/20 |
| Dastjerd | 3/10 | 3/10 | 6/20 |
| Total | 5/50 | 9/50 | 14/100 |

goat breeding farms were positive for *C. burnetii*. One of 70 (1.4%) camel bulk milk samples from 22 camel breeding farms was also positive for *C. burnetii* (Rahimi et al. 2011). In a similar work by Rahimi et al. (2009) in Chaharmahal va Bakhtiari province, 6.2% bovine milk samples and 1.8% caprine milk samples were positive (Rahimi et al. 2009a). The (14%) of C.Brunetti positive rate in our study is higher from other studies in other provinces in Iran. We have the highest rate of infection in summer. The findings of this study are limited to PCR-based detection of *C. burnetii* DNA in bulk milk samples, so we are unable to speculate the viability of organisms in milk samples, or the sensitivity and the specificity of the nested PCR assay compared to other diagnostic methods (Rahimi et al. 2009b). The result supports the hypothesis of high prevalence and endemic pattern of Q fever in Qom province. Finally, regarding the public health issue of Q fever, we suggest that serological and molecular surveys on other species of live stock be carried out in Qom province and other provinces of Iran to have a clear picture of *C.Brunetti* infection in Iran.

## Acknowledgments

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# تشخیص کوکسیلابرونتی با استفاده از آزمون زنجیره‌ای پلی مرازآشیانه‌ای در شیرهای فله‌ای گاو دارای‌های استان قم در سال ۱۳۹۰

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## چکیده

**زمینه مطالعه:** تب Q یک بیماری مشترک بین انسان و دام است که عامل آن، یک گونه از باکتری بنام *Coxiella burnetii* است که سراسر جهان در حال توزیع می‌باشد. در گاو، عفونت *Coxiella burnetii* معمولاً بدون علامت است اما همچنین می‌تواند با اختلالات تولید مثلی در ارتباط باشد. اپیدمیولوژی تب Q در ایران نا مشخص است اما برخی از منابع از اندمیک بودن ان در ایران خبر می‌دهند. **هدف:** هدف از این مطالعه به تشخیص مولکولی *Coxiella burnetii*، در مزارع گاوشیری با استفاده از واکنش زنجیره‌ای پلی مرازآشیانه‌ای در استان قم در سال ۱۳۹۰ می‌باشد. **روش کار:** از ژانویه تا فوریه ۲۰۱۱ (زمستان) و جولای تا سپتامبر ۲۰۱۱ (تابستان) در مجموع از ۱۰۰ نمونه بالک شیر گاو از پنج منطقه به یک تعداد جمع‌آوری شدند. **نتایج:** در این پژوهش، ۱۴٪ (۱۴ از ۱۰۰) شیر بالک مثبت بوده‌اند. **نتیجه‌گیری نهایی:** این نتایج فرضیه شیوع بالا و الگوی بومی تب Q در ایران را تایید می‌نماید.

**واژه‌های کلیدی:** کوکسیلابرونتی، واکنش زنجیره‌ای پلی مرازآشیانه‌ای

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