Entomology and Applied Science Letters
Volume 4, Issue 2, Page No: 11-15

Available Online at: www.easletters.com

ISSN No: 2349-2864

The Sensitivity of the Real-time PCR and Nested-PCR for Detection of *Coxiella burnetii* in Milk Samples

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DOI: 10.24896/easl2017423

ABSTRACT

Coxiella burnetii is the causative agent of Q-fever, a widespread zoonosis. In domestic animals infection remains either asymptomatic or presents as infertility or abortion. Clinical presentation in humans can range from mild flu-like illness to acute pneumonia and hepatitis. In humans serology is the gold standard for diagnosis but is inadequate for early case detection, so real-time PCR and nested-PCR assays were developed in this study to measure amounts of C.burnetii shed in milk. Our study was to assess the sensitivity of the real-time PCR and nested-PCR for detection of Coxiella burnetii in bovine bulk milk samples from dairy herds in 3 provinces (Chaharmahal and Bakhtiari , Isfahan and Yazd) of Iran. In the present study, 300 bulk milk samples from 89 dairy cattle herds were tested for C. burnetii using real-time PCR and nested-PCR assays. The animals which their milk samples collected for this study were clinically healthy. In total, 74 of 300 (24.7%) cow milk samples were positive in real-time PCR assay and 26 of 300 (8.7%) samples were positive in nested-PCR assay. McNemar test shows a significant difference in detection of C. burnetii between real-time PCR and nested-PCR. Also the results of this study indicate those clinically healthy dairy cows are important sources of C. burnetii infection in Iran.

Keywords: *Coxiella burnetii*, Real-time PCR, Nested-PCR, Milk, Iran.

HOW TO CITE THIS ARTICLE: Mojtaba Bonyadian, Hamdollah Moshtaghi, Hamidreza Kazemeini, The Sensitivity of the Real-time PCR and Nested-PCR for Detection of Coxiella burnetii in the Milk Samples, Entomol Appl Sci Lett, 2017, 4 (2):11-15,
DOI: 10.24896/easl2017423

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Received: 12/12/2016
Accepted: 25/03/2017

INTRODUCTION

Q fever is a ubiquitous zoonosis caused by Coxiella burnetii, an obligate intracellular rickettsial organism. Since the first independent reports by Australian and American investigators in 1935, Q fever has been found throughout the world, except New Zealand [1]. *C. burnetii* infections have been reported in humans, farm animals, pet animals, wild animals, and arthropods [2]. Q fever has been associated mostly with late abortion and reproductive disorders such as premature birth, dead or weak offspring, and endometritis and infertility in domestic ruminants [1, 14]. Infected animals usually shed the agent intermittently in milk, feces and urine, with no outward signs of disease, and should be regarded as possible sources of human infection [15]. In human beings, symptoms are highly variable and about 60% of infections are asymptomatic seroconversion patients. However, Q fever may lead to serious complications and even death in patients with acute disease. Predominant clinical manifestations are fever, pneumonia and granulomatous hepatitis for acute cases and endocarditis for chronic cases [1, 16, 17]. Among farm animals, dairy cattle, sheep and goats are the major reservoirs of *C. burnetii*. Animals are often naturally infected but usually do not show typical symptoms of *C. burnetii* infection. Clinical signs of *C. burnetii* infection are abortion in sheep and goats and reproductive disorders in cattle [1, 3]. *C. burnetii* can be isolated from the blood, lungs, spleen and liver of infected animals in the acute phase of the disease. The uterus and mammary glands are primary sites of infection in the chronic phase of *C. burnetii*. Shedding of *C. burnetii* into the environment occurs mainly during parturition by birth products, particularly the placenta of sheep. Also, shedding of *C. burnetii* in milk by infected dairy cattle is well

documented [1, 3].This bacterium is very stable in different environments. It is also highly infectious and one to ten organisms can cause Q fever in humans [5]. Also, *C. burnetii* can be present in milk, urine, feces, vaginal mucus and semen. In milk, it can be secreted for 8 days in ewes and up to 13 months in cattle [6]. The consumption of contaminated raw milk does not seen to represent an efficient route of disease transmission, however bulk milk samples is an important specimen for epidemiological survey on dairy herds [7]. In order to management, prevention, control and treatment of Q fever in animal and human, early and accurate detection of *C. burnetii* is very necessary. Serological methods have been used to detect antibodies to *C. burnetii* [18, 19, 20]. These assays may not be useful for the diagnosis of acute infection due to the delay in antibody development. Furthermore, it is difficult to discriminate between current and past infection because antibodies often persist after the organisms disappear from the blood [17]. PCR assay has become a useful tool for the detection of *C. burnetii* in clinical samples because of the low detection limit and high sensitivity [15, 17, 21, 22, 23]. This assay has been described as the most sensitive and rapid means to identify shedder animals [16]. Previous studies on the prevalence of *C. burnetii* in dairy cows were based mainly on serologic tests that detect antibodies that could have been introduced months earlier [8]. Recently, PCR has been used to detect *C.burnetii*. PCR is a safe, sensitive and specific method for the detection of *C. burnetii* in different samples [9]. Real-time PCR and nested-PCR assays targeting IS1111 was developed in this study to identify *C. burnetii* shed in milk. Our study was to assess the sensitivity of the real-time PCR and nested-PCR for detection of *C. burnetii* in bovine bulk milk samples from dairy herds in 3 provinces (Chaharmahal and Bakhtiari, Isfahan and Yazd) of Iran.

MATERIALS AND METHODS

In this cross-sectional study 300 bovine bulk milk samples were collected randomly from dairy herds of three provinces included Chaharmahal and Bakhtiari (n= 100), Isfahan (n=100) and Yazd (n=100) in Iran during a year period from August 2014 to July 2015. The samples were collected in four season and immediately transported to the laboratory and were tested. One mL of raw milk was centrifuged. This procedure was performed to isolate the bacterial cells in pellet of the milk samples. After removing the cream and milk layers [12], DNA was extracted from the pellet by a genomic DNA purification kit (Cinna Gen Co., Iran) according to the manufacturer's protocol [13]. DNA samples were stored at-20ºC until they were used. For real-time PCR using Rotor-Gene system (Corbett, Australia) and the following primers and probes:

Cox-F2: 5'- AAAACGGATAAAAAGAGTCTGTGGTT -3' TM: 69.4
Cox-R2: 5'- CCACACAAGCGCGATTCAT -3' TM: 66.3
Cox-probe: FAM- AAAGCACTCATTGAGCGCCGCG – TAMRA TM: 78.5
Size: 69 bp
Color: Green
Gene Bank: LK937696.1

Statistical analysis

All data were analyzed by SPSS (version 16; SPSS Inc., Chicago, IL, USA). The X^2 and Mcnemar test was used to identify statistically significant associated with presence of *C. burnetii* in bulk milk samples. The level of statistical significance was $P<0.05$.

RESULTS AND DISCUSSION

In this study, a total of 300 samples of raw milk were collected from dairy herds of three provinces included Chaharmahal and Bakhtiari, Isfahan and Yazd and to detect *C. burnetii* using nested polymerase chain reaction were tested. Length of specific band was 501 bp and primer binding temperature set on 58 ° C (Figure 1).

The results of observing samples with *C. burnetii* infection during the first phase Nested-PCR

A total of 26 samples (8.7%) were contaminated with *C. burnetii* (Figure 2). Of which 14 (14%) were samples of Chaharmahal and Bakhtiari province; 7 (7%) samples of Isfahan and 5 (5%) samples of Yazd province. Statistical tests show a significant difference between contamination rate in the province of Chaharmahal and Bakhtiari compared with Isfahan and Yazd provinces ($P <0.05$); (Table 1). According to the results, the prevalence of *C. burnetii* in raw milk in different seasons was different so that in winter and spring seasons is more than other seasons ($P <0.05$).

Results of Real-time PCR

According to this method, 74(24.7%) of 300 milk samples were infected with *C. burnetii* which 33 (33%) samples were in Chaharmahal and Bakhtiari province, 26 (26%) samples in Isfahan and 15 (15%) samples in Yazd province. Statistical test show a significant difference

between contamination rate in the province of Chaharmahal and Bakhtiari and Isfahan compared with Yazd provinces (P <0.05); (Table 2). According to the results, the prevalence of *C. burnetii* in raw milk in different seasons was different so that in winter and spring seasons is more than other seasons (P <0.05).

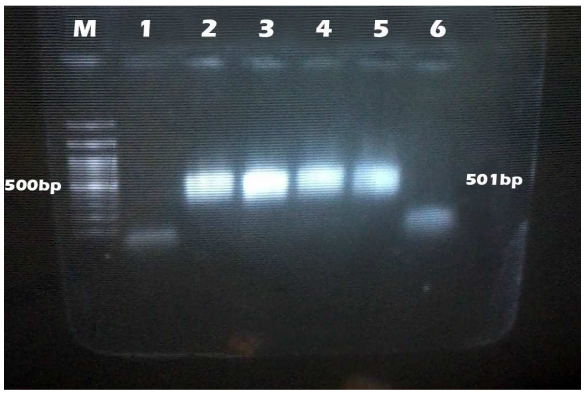

Figure 1: M: Marker 100 bp made by Sina Gene Company, lanes 2 to 6: Products PCR at first phase, lane 1: negative control

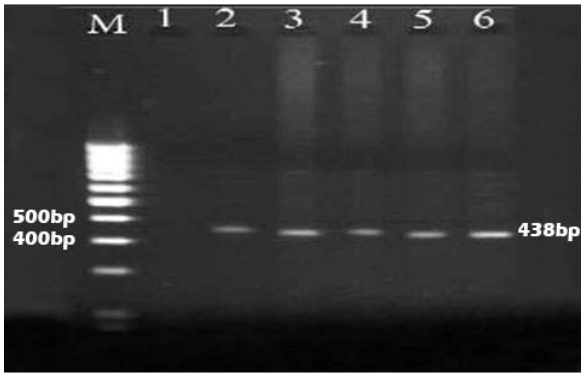

Fig. 2: The PCR-amplified products. Lane M 100 bp DNA ladder; lane 1 positive control; lanes 2 and 3 positive samples of *C. burnetii*; 4 negative control. The lanes show the amplification of a 438-bp fragment in the com1 gene of *C. burnetii*.

Comparing both NestedPCR and Real-time PCR test result

According to Nested-PCR test results from a total of 300 samples of raw milk taken, 26 (8.7%) samples were contaminated with *C. burnetii* but the number of infected samples in real- time PCR was 74 (24.7%) samples. McNemar test showed that the difference between the two test results were not statistically significant (P <0.05). Also agreement index between the two tests (K) is 0.16 that represent poor agreement between the results of two tests. Nested-PCR and Real Time PCR product with primers and probes used in this study to Takapouzist Company and homology of it was studied by *C. burnetii*, which 100% was consistent. The study aim was to comparison of real-time PCR and nested-PCR in sensitivity for detection of *C. burnetii* and associated risk factors in dairy bovine herds. There is no previous study on the sensitivity of detection for *C. burnetii* existed in this region by real-time PCR and nested-PCR. Bulk tank milk has been used for surveillance samples in dairy herds for several bovine diseases including bovine viral diarrhea. In a study conducted in Switzerland, 4.7% of bovine bulk milk samples via nested PCR tested positive for *C. burnetii*[10]. Another study in the USA reported a prevalence of *C. burnetii* over 94% in bulk milk samples from dairy herds. It was carried out during a 3-year period from January 2001 to December 2003 by trans-PCR [11]. Rahimi et al assessed the prevalence rate of *C. burnetii* in bovine, ovine, and caprine bulk milk samples by nested PCR in Chaharmahal and Bakhtiari province of Iran. In their study, 6.2% of bulk milk samples tested positive for *C. burnetii* while all ovine bulk milk samples were negative for *C. burnetii*. Also, 1.8% of tested caprine bulk milk samples were positive. They reported that clinically healthy cattle are the main sources of *C. burnetii infection* in Iran [8]. In another study, Rahimi et al. tested bulk milk samples from dairy goat herds in five different provinces of Iran for *C. burnetii* via nested PCR. It was found that 18.2% of herds in Fars, 5.5% in Yazd, and 4.2% in Khuzestan provinces tested for *C. burnetii* were positive. All of the samples in the Qom and Kerman provinces were negative [7]. In another study they analyzed the prevalence rate of *C. burnetii* in bulk milk samples from dairy bovine, ovine, caprine, and camel herds via nested PCR in the center of Iran. The prevalence rate of *C. burnetii* in bovine, ovine, caprine, and camel bulk milk samples were 3.2%, 5.7%, 4.5%, and 1.4%, respectively [8]. Food can play an important role in animal contamination. A wide range of pathogens such as *Listeria spp., Escherichia coli*, and *Bacillus cereus*, have been associated with dairy cattle feed. Does *C. burnetii* belong to this set? In these herds tap water and pit water were used as sources of water and river water was not used. Other studies reported several causes of *C. burnetii* infection relevant to river and pond water. Burganskii et al reported a “Q fever” outbreak in the Urals in test results of river water that was the source of water for bovine herds [5]. Whitney et al. reported that veterinarians who are in contact with water, especially pond water, were more likely *C. burnetii* to be positive [4]. Welsh et al. observed *C. burnetii* in pools water sampling in the lambing areas on ranches in USA. They also found *C. burnetii* in pools of water 1 month later [6].

Table 1: Nested-PCR test results of the bulk tank samples

Season Province	Number	Spring	Summer	Fall	Winter	Total
Chaharmahal and Bakhtiari	100	4	2	2	6	14(14%)
Isfahan	100	2	1	1	3	7(7%)
Yazd	100	2	0	1	2	5(5%)
Total	300	8(10.7%)	3(4%)	4(5.3%)	11(14.7%)	26(8.7%)

Table 2.Real-time PCR test results of the bulk tank samples

Season Province	Number	Spring	Summer	Fall	Winter	Total
Chaharmahal and Bakhtiari	100	10	3	5	15	33(33%)
Isfahan	100	8	2	4	12	26(26%)
Yazd	100	4	2	2	7	15(15%)
Total	300	22(29.3%)	7(9.3%)	11(14.7%)	34(45.3%)	74(24.7%)

Kulagin et al reported that *C. burnetii* can survive in tap water for a long time [3]. Therefore water exposure with an environmental investigation should be considered in future studies. Another analyzed variability regarding the kind of dairy bovine herd and the presence of *C. burnetii* in bulk milk samples. All of the positive samples were found in rural dairy herds, but the difference was not statistically significant. The presence of *C. burnetii* in rural herds can be a threat for human health because of the presence of herds close to human settlements. The only association was found in the samples were taken in season. Most of the positive samples were in winter. This result is similar to another study in Iran which showed the highest incidence of *C. burnetii* in winter. All milk samples collected in the summer, were negative for *C. burnetii* [2]. Fretz et al also reported a highest incidence of *C. burnetii* in the winter [1]. It may be due to the calving period of bovine that is more often found in the winter. These results oppose with Rodolakis et al who reported a highest incidence of "Q fever" in the spring and summer [3]. Different results for the incidence of *C. burnetii* in different regions may be the cause of different geographic properties, used test (serological or molecular test), kind and number of tested samples, and season. Infected animals can also shed bacteria by other routes such as feces, urine, placenta and vaginal mucus. Therefore it is better to screen large numbers and various types of samples in order to obtain correct results of the animal's condition. Also, more studies are essential to fully recognize the epidemiology of *C. burnetii*. This study proposes that those people who contact with animal, milk and dairy products must be tested for *C. burnetii* infection. In the future, research regarding environmental surveillance should be considered. Also, more investigations are needed to recognize effective factors on environmental survival of *C. burnetii*.

CONCLUSION

According to the infection caused by the presence of *C. burnetii* in dairy products supplied to the market of Chaharmahal and Bakhtiari, Yazd and Isfahan provinces, shows the role of pathogenic bacteria with infectious dose is very low for serious diseases such as endocarditis and meningitis, preventive measures against the spread of the bacteria in livestock and their by-products to be pursued more seriously. Since, the amount of contamination in products on the market was not low; due to the high heat resistance *C. burnetii* consumption of traditional and unpasteurized dairy products is not recommended.

Acknowledgment
The authors would like to thank Dr. Abbas Doosti for the sincere help in performing technical parts of the project.

Funding
The present paper has been developed under the cooperation and financial supports of Vice chancellor in research and technology, Shahrekord University, Shahrekord, Iran.

Authors Contribution
M Bonyadian, H Moshtaghi and H.R Kazemeini designed the study, developed the methodology, collected the data, performed the analysis, and wrote the manuscript.

Conflict of Interest
There are not any conflicts of interest in this manuscript.

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