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Detection of *Coxiella burnetii* in raw milk samples collected from dairy farms in Mazandaran province, north of Iran

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ABSTRACT

Query fever is an important zoonotic disease caused by the obligate intracellular microorganism of *Coxiella burnetii*. Moreover, the present study aimed to determine the prevalence of *C. burnetii* in bulk milk samples from dairy bovine herds using a nested polymerase chain reaction assay. During four seasons, a total of 100 samples of bulk milk from traditional, semi-industrial, and industrial dairy bovine herds located in six cities of Mazandaran province, northern Iran from May 2019 to February 2020 were collected. The samples were subjected to detect *C. burnetii* using a nested-PCR assay. Twenty - seven out of 100 bulk milk samples (27%, 95% CI: 18.3-35), 7% were infected with *C. burnetii*. The prevalence was 54.3% (19 samples) in traditional dairy herds and 12.3 % (8 samples) in semi-industrial industrial dairy herds. Furthermore, our results revealed that the chance of milk being infected with *C. burnetii* bacteria in traditional milk is 8.5 times higher than the milk of semi-industrial and industrial dairy herds. Based on the obtained results of the current study, the highest and lowest prevalence of *C. burnetii* were seen in Ghaemshar (66.7%) and Babol (0%), respectively ($p<0.05$). Spring season (37%) had the highest contamination of *C. burnetii* in bulk milk samples. According to the findings of this study, the clinically healthy dairy cows are important sources of *C. burnetii* infection in this area, amplified by the traditional herds of these animals. Because these animals can transmit the infection to humans, this common zoonotic bacterium can be a potential health problem in Mazandaran province, Northern Iran.

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1. Introduction

Query fever is a zoonotic disease with almost worldwide distribution caused by *C. burnetii*. An obligatory intracellular gram-negative bacterium, small coccobacillus (0.4 to 1 µm long and 0.2 to 0.4 µm wide), not stainable with the Gram technique and belonging to the family of *Coxiellaceae* (1). *C. burnetii* is considered a common zoonotic contaminant because it can cause infections in a wide range of animals, from arthropods to humans, such as wild and domestic animals, birds, and reptiles (2).

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Cattle, sheep, and goats are the primary reservoirs of human infection. Nevertheless, this ubiquitous zoonotic bacterium can infect many animal species (3). Both symptomatic and asymptomatic infected animals shed large amounts of *C. burnetii* into the environment. *C. burnetii* is mainly excreted during and after parturition or abortion in birth products (placenta and delivery fluids) as well as in the urine, feces, and vaginal secretions of infected animals (4). However, the consumption of milk and dairy products, skin contact, and person-to-person transmission are the other transmission routs of the infection (5).

Query fever is generally regarded as an occupational hazard, and direct contact with ruminants may be strongly associated with the disease in humans (6). This widespread outbreak, as well as the worldwide epidemic, has highlighted Q fever as a reemerging threat to public health. Moreover, the high distribution of *C. burnetii* in productive animals necessitates the evaluation of the pathogen presence in animal origin foods and the associated potential risk to public health. It is important to note that unpasteurized raw milk is the most important source of *C. burnetii* among foods of animal origin (7). The pathogen is excreted in the milk of infected animals with clinical signs of infection or during variable periods during lactation and can be isolated. The presence of *C. burnetii* in milk raises concerns about the role of unpasteurized raw milk or unpasteurized raw-milk products as possible routes of this common zoonotic bacterium into humans. In order to prevention, management, control, and treatment of Q fever in animals and humans, early and accurate detection of *C. burnetii* is very necessary. Based on different studies, Q fever is known as an endemic disease in Iran and recently acute and chronic cases of Q fever have been reported in different parts of the country (3,6,8). On the one hand, due to the intracellular nature of *C. burnetii*, it cannot be cultured by standard culture methods. On the other hand, the time required to produce antibodies against this pathogen (which may take several weeks) is an important drawback of using serological tests to diagnose acute infection (8). As a result, molecular techniques including PCR have been proposed as rapid and sensitive methods to determining *C. burnetii* infection in most ruminants (9). At the moment, we don't have any information on the prevalence of *C. burnetii* in raw milk in northern Iran. Therefore, the present study aimed to determine the prevalence rate of *C. burnetii* as a milk-borne pathogen in bulk milk samples from dairy bovine herds in Mazandaran province, northern Iran.

2. Materials and Methods

2.1. Study regions

This research work was carried out in the province of Mazandaran (This province with Green pastures and abundant industrial herds and traditional herds has an important role in the production of dairy products in Iran), north of Iran, during which bovine samples were collected from the western, central and eastern regions of the province. In each region, cities were randomly selected, including Amol, Babol, Ghaemshahr, Jouybar, Mahmoodabad, and Noor.

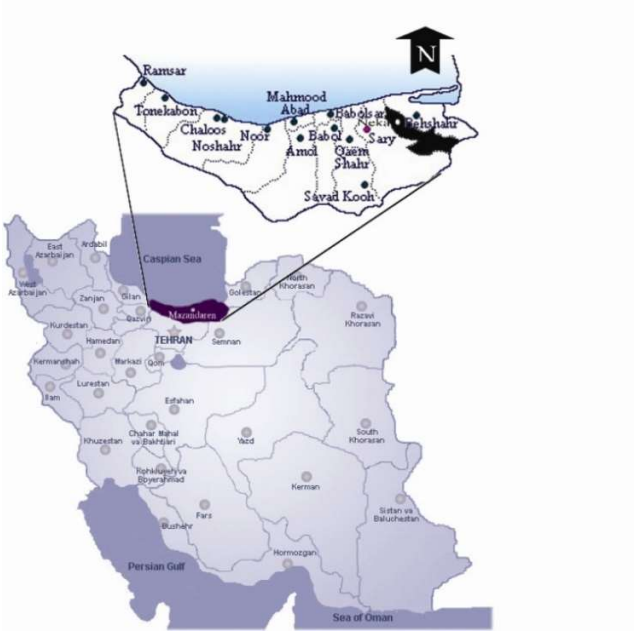

Figure 1. Map of Iran, highlighting the position of Mazandaran Province, north of Iran

2.2. Sample collection

To investigate in this research work, because of the possibility of the effect of climate on the prevalence of this disease is possible, 2-stage cluster sampling was performed to have samples from all over the province and to be able to calculate the real prevalence of the disease. The different cities of Mazandaran province were divided into three clusters: east, west, and center, and in each cluster, cities were randomly selected and many farms were randomly selected from the selected cities. The sample size was calculated using the sample size formula for cross-sectional studies. The prevalence of *Coxiella burnetii* in raw cow milk samples was estimated to be 7, the confidence level was 95% and the precision was 5% equal to 100 samples.

Then, the sample size was divided according to the livestock population of each city and many samples were randomly taken from each city. A total of 100 bulk milk samples were collected from 65 semi-industrial and industrial herds and 35 traditional herds, located in different cities of Mazandaran province. All milk samples were collected according to sampling principles from May 2019 to February 2020. It should be noted that in this study the *C. burnetii* infection status of the herds was unknown before this study, and there was no established surveillance or management targeting *C. burnetii* control. No vaccination for *C. burnetii* was performed on these herds. On the one hand, the animals whose milk samples were collected for this study were clinically healthy and the milk samples showed physical (color, pH, and density) consistency. The samples were immediately transported to the laboratory in a cooler with ice packs and were processed within one h of collection.

2.3. DNA extraction and nested PCR reaction

For this purpose, after centrifugation and removal of the fat and milk layers, DNA was extracted from the pellet by a genomic DNA purification kit (*Cinna Gen Co., Iran*) according to the manufacturer's instructions. DNA samples were stored at −20°C until used. In this research, we used primers designed from the *com1* nucleotide sequence of the *com1* gene, which encodes a 27-kDa outer membrane protein (OMP) as described previously (10). Two primer pairs OMP1, OMP2, OMP3, and OMP4 in this study were obtained from a commercial source (*CinnaGen Co, Iran*) (Table 1).

The PCR was performed at 94°C for 4 min and then for 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min in DNA thermal cycler (Master Cycler Gradient). In the second amplification, the reaction was performed in a total volume of 25 µl including 2 µl of DNA sample, 0.5mM $MgCl_2$, 0.2mM (each) dNTPs, 0.8 mM primer OMP3, 0.8 mM primer OMP4, and 0.5U = reaction of Taq DNA polymerase. The PCR was performed at 95°C for 4 min and then for 30 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Products amplified by PCR (OMP1-OMP2, 501 bp; OMP3-OMP4, 438 bp) were examined by electrophoresis in 1.5% agarose gel, stained with 1% ethidium bromide solution and under UV light (10).

2.4. Statistical analysis

In this study, all data were analyzed using SPSS software (*version 16; SPSS Inc., Chicago, IL, USA*). A Chi-square test was used to identify statistically significant factors associated with the presence of *C. burnetii* in bulk milk samples. Data analysis was done using SPSS statistical package (version 25.0) (SPSS Inc., Chicago, IL, USA). For all analyses, ($p<0.05$) was considered statistically significant.

3. Results

C. burnetii may be widely prevalent but undiagnosed in livestock in Iran. As mentioned, the diagnosis of "query fever" in animals and humans is important for prevention, management, control of disease, as well as early and accurate detection of *C. burnetii* is required. Molecular assays can detect microorganisms and confirm the shedding of bacteria and thus the current infection (11).

In this research, a total of 100 bulk milk samples from 60 dairy bovine herds in Mazandaran province were tested for *Coxiella burnetii* using a molecular method. Nested PCR assay showed a specific band with a molecular weight of 501 bp during the first phase (Fig. 2).

Figure 2. M: Ladder 100 bp; lanes 2 to 6: PCR products at first phase; lane 1: negative control.

Amplification showed a band with 438 bp, indicating the expected length for detection of *C. burnetii* in the second phase of nested PCR (Fig. 3).

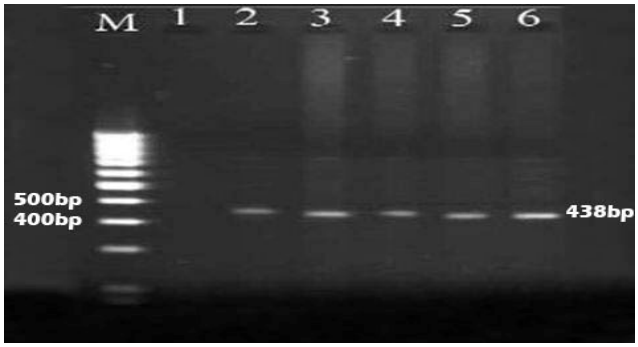

Figure 3. PCR-amplified products. M: 100 bp DNA ladder; lane 1: positive control; lanes 2 and 3: positive samples of C. burnetii; lane 4: negative control. The lanes indicate amplification of a 438-bp fragment in the C. burnetii com1 gene.

Out of the 100 milk samples, 27 (27%) were positive using nested-PCR. The results showed that the highest prevalence of *C. burnetii* was seen in Joybar (100%, p<0.05), followed by Mahmoodabad (66.7%), Joybar (7.4%), Ghaemshahr (40%), and Noor (25%) cities. On the one hand, all collected samples from Babol city were also negative for the presence of *C. burnetii*. Our results revealed that the prevalence of ubiquitous zoonotic bacterium (*Coxiella burnetii*) in bovine milk samples from Mazandaran province (27%) is higher than in other provinces of Iran. Table 1 shows the number of positive samples in different seasons of the year. As can be seen, the relationship between the season and the prevalence of *Coxiella burnetii* is not significant, so the calculated odds ratio is also not significant.

Table 1. Prevalence of *Coxiella burnetii* in bulk milk from dairy bovine herds in Mazandaran province based on a different season

Season	**Positive sample**	**Negative sample**	**[b]OR (95% CI)**	**[c]P-value**
Spring[a]	10 (37 %)	15 (20.5 %)		
Summer	6 (22.2%)	19 (26%)	0.47 (0.14-1.60)	0.22
Autumn	6 (22.2%)	19 (26%)	0.47 (0.14-1.60)	0.22
Winter	5 (18.5%)	20 (27.4%)	0.37 (0.10-1.32)	0.12

[a]Reference group. [b]Odds ratio (confidence interval for OR). [c]P<0.05 was considered statistically significant.

Table 2 shows the positive sample numbers based on types of herds. As can be seen, the relationship between herd type and the prevalence of *Coxiella burnetii* is significant and the chance of milk being infected with *Coxiella burnetii* bacteria in traditional milk is 8.5 times (OR= 8.50; 95% CI= 3.13 to 22.88; P< 0.001) higher than the milk of semi-industrial and industrial dairy herds.

Table 2. Prevalence of *Coxiella burnetii* in bulk milk from dairy bovine herds in Mazandaran province based on types of herds

Types of herds	Positive sample	Negative sample	[b]OR (95% CI)	[c]P-value
Semi industrial and Industrial[a]	8 (12.3%)	57 (87.7%)		
Traditional	19 (54.3 %)	16 (45.7 %)	8.50 (3.13-22.88)	0.001

[a]Reference group. [b]Odds ratio (confidence interval for OR). [c]P<0.05 was considered statistically significant.

As can be seen in Table 3, the highest number of samples was from Ghaemshahr city, which is due to the proportion of livestock population in this city. The studied cities showed a significant difference in terms of the frequency of positive samples to *Coxiella burnetii* (p < 0.001). The highest frequency of positive samples was seen in Joybar, Mahmood Abad, and Ghaemshahr cities, which were equal to 100, 66.7, and 40%, respectively.

Table 3. Prevalence of *Coxiella burnetii* in bulk milk from dairy bovine herds in Mazandaran province based on different cities

City	Number of samples	Positive sample	Negative sample
Babol	20	0 (0 %)	20 (100.0 %)
Amol	22	3 (13.6%)	19 (86.4%)
Joybar	2	2 (100.0%)	0 (0%)
Mahmoodabad	3	2 (66.7%)	1 (33.3%)
Noor	8	2 (25.0%)	6 (75.0%)
Ghaemshahr	45	18 (40.0%)	27 (60.0%)
Total	100	27 (27 %)	73 (73%)

Chi square= 21.71, P- value<0.001

4. Discussion

Recent studies have reported a global prevalence of *C. burnetii* in bovine milk of 15.1% (95% CI=11.08-19.10) by PCR (12). The prevalence of *C. burnetii* in bovine milk has been reported from 11 provinces of Iran. According to reports, the highest and lowest prevalence of Q fever were observed in East Azerbaijan (25.6%) and Khorasan-Razavi (4.2%) provinces, respectively (13,14). Also, the results of other Iran's provinces were as follows: 12% in Tehran (8), 14% in Qom (15), 6.2% in Chaharmahal-Va-Bakhtiari (16), 13.2% in Zanjan (17), 5% in Yazd (18), 11% in Fars (3), and 3.2% in Isfahan (19). The molecular data evaluated in this study showed that the overall prevalence of *C. burnetti* infections in different regions of Iran varies from one region to another.

This heterogeneity in prevalence may be due to differences in sample sizes, design of the studies, detection method (PCR, nested-PCR, and Real-Time PCR) and sampling season (3). In addition, it may reflect agricultural or climatic differences between different regions in our country, but what is important the prevalence of *C. burnetii* in milk poses this bacterium as an important health hazard in Iran. Therefore, milk shedding in cows is one of the most common ways of spreading *C. burnetii* into the environment. This is an important point that can be considered in the prevention and control function of Q fever in animals. Different genotypes of *C. burnetii* are circulating in different hosts (goat, sheep, and cow) and therefore, molecular typing and genotyping studies are recommended to confirm the main source of human infections in Iran.

Evaluation of the prevalence of *Coxiella burnetii* in other countries shows different levels of prevalence in bovine milk, for example, 4.7% in Switzerland (20), 8.7% in Hungary (21), 18.8% in the Netherlands (22), 22% in Egypt (23), 14.3-40% in Italy (24), 28.9% in Saudi Arabia (25), 42.9% in the USA (26) and 17.8% in South Korea (27). These studies have shown that *C. burnetii* shedding in milk is widespread in dairy bovine herds in different parts of the world and interpretation of these results in various countries is difficult, because of the dairy cattle's age, various assessing methods, and different epidemiological circumstances (12).

In the present study, 19 positive samples were related to traditional dairy herds, while only 8 samples from industrial dairy herds had positive results.

Statistical analysis showed that the infection rate in traditional and industrial dairy herds was 54.3% and 12.3%, respectively. There was a significant difference in the level of contamination with *C. burnetii* between raw milk samples from different dairy herds ($p<0.05$). Similar results were reported in studies by Nokhodian et al. (2016) and Rahmdel et al. (2018) (28,29). In general, evaluation results of milk samples collected from traditional dairy herds indicate a higher prevalence of bacteria than industrial samples, which may be due to non-standard farm construction (such as ventilation and waste management) and unsanitary practices (e.g. poor husbandry and breeding conditions). In addition, most traditional bovine dairy farms in Iran, especially in Mazandaran province, sell milk in form of unpasteurized for consumption in supermarkets. Unfortunately, this tradition of consuming dairy products from unpasteurized milk, especially among rural residents and remote areas of Mazandaran province, increases the risk of diseases caused by pathogens through milk.

The obtained results of the current study also demonstrated that the highest and lowest prevalence of *C. burnetii* occurred in the milk samples taken from spring (37%) and winter (18.5%) seasons, respectively (Table 1). That doesn't show statistical significance. Abdali et al. (2018) also reported the highest prevalence of *C. burnetii* in spring (30). Our results contradict Kargar et al. (2013) and Fretz et al. (2007) who reported the highest prevalence of *C. burnetii* in winter (3,20). This difference in the results may be due to the calving period of bovine, which is mostly seen in the spring in Mazandaran province of Iran.

5.Conclusion

According to the findings of this study, the prevalence of ubiquitous zoonotic bacterium (*Coxiella burnetii*) was very high and considerable (27%) in bulk milk samples from cattle in Mazandaran province compared to other provinces of Iran. Therefore it can be said, necessary measures should be taken to increase awareness of preventive methods such as pasteurization of milk and milk products, use of personal protective equipment, and appropriate control in dealing with livestock.

Conflict of interest

The authors declare they have no conflict of interest.

Acknowledgments

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