



Prevalence of *Coxiella burnetii* in Traditional and Industrial Butter and Cream Using Nested Polymerase Chain Reaction in Shahrekord, Iran

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Abstract

Background: A zoonotic disease of global importance known as “Q fever” is caused by *Coxiella burnetii* agent. The aim of the present research was to determine *C. burnetii* in the traditional and industrial butter and cream samples in Shahrekord, Iran.

Methods: This cross-sectional descriptive study was conducted from March to September 2016. Totally, 200 traditional and industrial butter and cream samples were collected from retailers in different regions of Shahrekord and then tested for *C. burnetii* via nested polymerase chain reaction (PCR) assays.

Results: In this survey, a total of 6 out of 200 samples (3%) were found to be positive for *C. burnetii*. More precisely, 4 (5.79%), 1 (5%), and 1 (2.56%) samples were related to the traditional bovine cream, traditional sheep butter, and traditional bovine butter samples, respectively. Nevertheless, no *C. burnetii* infections were found in the industrial butter and cream samples.

Conclusions: These results proved that traditional dairy products can be considered as an important reservoir for *C. burnetii* infection in Iran.

Keywords: *Coxiella burnetii*, Industrial butter, Industrial cream, Nested PCR, Iran

Background

Coxiella burnetii is an agent of zoonosis diseases that occur in humans and animals. “Q fever” is the disease that is caused by *C. burnetii* and coxiellosis, and affects humans and animals (1). *C. burnetii* as a small gram-negative bacterium is morphologically similar to *Rickettsia* although they are genetically and physiologically different (2). Genus *Coxiella* has different species while *C. burnetii* is the only species of *C. burnetii* formed via *16s-rRNA* gene sequencing, (2). The bacterial infection is carried in the environment and transmitted by ticks which are the principal vectors of the bacterium. There can be several routes of infection in humans, including oral transmission via contaminated raw milk and dairy products although the inhalation of contaminated aerosols stands on top (3).

The infection can also be spread through the milk, fecal material, urine, vaginal secretions, especially at the time of abortion or parturition of the animals which are infected with *C. burnetii* (1). Q fever most commonly occurs in people who are working in the livestock industry including animal breeders, slaughterhouse workers, dairy industry

staff, leather factory workers, or/and oil and industries that involve the transportation of the animals (4). This fever is often diagnosed only through a systematical consideration since its clinical manifestations are highly variable. In addition, only serology would provide the best approach to the distinction between acute and chronic infections. Further, Q fever in humans is usually manifested with no symptoms or as a mild flu-like disease which can recover spontaneously. However, some patients represent more serious states of the disease, some of which may lead to death. The risk factors of chronic disease include cardiac valve defects and vascular diseases, especially in immunocompromised hosts and pregnant women (3). According to Singh et al (5), the available methods for *C. burnetii* detection are as follows:

- Classical methods, for example, culturing microorganisms in suitable cell lines;
- Serological tests such as immunofluorescence, micro-immunofluorescence, complement fixation test, or the enzyme-linked immunosorbent assay (ELISA);
- Molecular biology techniques like nested and real-

time polymerase chain reaction (PCR).

Generally, dangerous and time-consuming *Coxiella* culturing is no longer performed for Q fever diagnosis since it is difficult. Hence, the use of diagnostic methods that are based on molecular biology techniques can significantly help the early diagnosis and treatment management of Q fever (6).

Objectives

No studies have so far investigated the role of cream and butter in *C. burnetii* infection with respect to Iran, and the rest of the world. Considering the low infectious dose of this pathogen and the lack of the availability of published data on the contamination of dairy products, the present study sought to evaluate the prevalence of *C. burnetii* in traditional and industrial butter and cream by nested PCR in Shahrekord, Iran.

Materials and Methods

Sampling

In total, 200 samples were collected from retailers located in different regions of Shahrekord, Iran during March–September 2016 that included: traditional cream samples (i.e., 10 goat, 20 sheep, and 39 bovine cream samples); traditional butter samples (i.e., 12 sheep and 69 bovine butter samples); industrial cream samples (25 bovine samples); and industrial butter samples (25 bovine samples). A cooler with ice packs was utilized to immediately transport the samples to a laboratory and process them in 1 hour after collection.

Using PCR to Definitely Diagnose *Coxiella burnetii* Zhang et al and Fretz et al methods were utilized to detect *C. burnetii* in the samples (7,8). Then, 2 g of butter or 2 mL of cream was transferred to the tube by using a genomic DNA purification kit (Cinnagene, Iran), followed by performing the PCR with the purified extracted DNA. The quality and quantity of the extracted DNA were evaluated by spectrophotometry with a ratio of 260-280 nm wavelength (7,8).

The nested PCR was applied to detect the presence of the *C. burnetii* genomic DNA in the samples. In addition, the methods of Zhang et al and Fretz et al (7,8) was also employed for the sequences of the primers that were used for the *com1* gene amplification (encoding the outer membrane of *C. burnetii*) (Table 1). Further, the optimum concentrations of substances with a final volume of 25 mL containing 5 µL of DNA sample, 0.5 mM MgCl₂, 1 µM primer OMP1, 1 µM primer OMP2, 0.5 U/reaction of Taq DNA polymerase, and 0.2 mM (each) dNTPs (Fermetas, Germany) were used to perform the first stage of the PCR (9).

Furthermore, sterilized mineral oil (2–3 drops) was added to the PCR mixture in order to avoid contamination and evaporation. Next, the tubes were placed in an Eppendorf Master Cycler Gradient Thermal

Cycler (Germany) and a temperature program was run for the PCR assay at 94°C for 4 minutes, and then for 30 cycles at 94°C for 1 minute, 56°C for 1 minute, and finally, at 72°C for 1 minute in a DNA thermal cycler (9). For the second amplification, the reaction was performed with a total volume of 25 µL including 2 µL of the DNA sample, 0.5 mM of MgCl₂, 0.2 mM (each) of dNTPs, 0.8 µM of OMP3, 0.8 µM of OMP4 primer, and 0.5 U/reaction of Taq DNA polymerase. The PCR assay was performed at 95°C for 4 minutes and then for 30 cycles at 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute. The PCR-amplified products (i.e., OMP1-OMP2: 501 bp & OMP3-OMP4: 438 bp) were examined by electrophoresis in a 1.5% agarose gel, stained with 1% solution of ethidium bromide, and then investigated under UV illumination. In the present study, the *C. burnetii* DNA (Serial Number: 3154; Genekam Biotechnology AG, Germany) and DNase- free water were used as positive and negative controls, respectively (9).

Statistical Analysis

Data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp, Redmond, WA, USA) SPSS software, version 19.0 (SPSS Inc., Chicago, IL, USA) and the chi-square test was applied to analyze the data. The differences were considered significant at $P < 0.05$.

Results

A number of 200 traditional and industrial butter and cream samples were experimented in Shahrekord and were reported to have *C. burnetii* using the Nested PCR (Table 2 and Figure 1). In total, 4 out of 69 (5.79%) traditional bovine cream samples, 1 out of 20 (5%) traditional sheep butter samples, 1 out of 39 (2.56%) traditional bovine butter samples were positive for *C. burnetii*. The statistical analysis of the obtained results revealed no significant difference between the various samples and the prevalence of *C. burnetii* ($P < 0.05$).

Discussion

Overall, the results of this study demonstrated the highest and lowest rates of infection in the traditional bovine cream samples (4 out of 69 samples, 5.79%) and traditional bovine (1 out of 39, 2.56%) and sheep (1 out of 20, 5%) butter samples, respectively. Our findings could be compared with those of several other studies conducted in Iran and other countries.

For example, Muskens et al reported that 78.6% of the cow milk samples contained antibodies against *C. burnetii* and the prevalence of *C. burnetii* in the samples tested was found to be 56.6% using the PCR (10). The most important reasons for the difference in the prevalence of *C. burnetii* in cow milk samples across the different regions of the world are related to various types of

Table 1. Primers for the Identification of *C. burnetii*

Primer	Sequence (5' → 3')	Amplicon Size (bp)
OMP1	5'-AGT AGA AGC ATC CCA AGC ATT G -'3	501
OMP2	5'-TGC CTG CTA GCT GTA ACG ATT G -'3	
OMP3	5'-GAA GCG CAA CAA GAA GAA CAC- '3	438
OMP4	5'TTG GAA GTT ATC ACG CAG TTG-'3	

Table 2. The Prevalence of *Coxiella burnetii* in the Samples of Traditional and Industrial Cream and Butter in Shahrekord

Samples	No. of Samples	No. (%) of <i>Coxiella burnetii</i> Positive Samples	
Traditional cream	Sheep	12	0 (0)
	Bovine	69	4 (5.79)
Industrial cream	Bovine	25	0 (0)
	Goat	10	0 (0)
Traditional butter	Sheep	20	1 (5)
	Bovine	39	1 (2.56)
Industrial butter	Bovine	25	0 (0)

geographic regions, the types of study methods, the type and the number of samples, the season of the year, as well as the sampling of infected and non-infected herds (11).

Similarly, Can et al used PCR with primers based on a repeat region, including transposon, for the direct detection of *C. burnetii* in the milk samples. In their study, 150 milk samples (9%) were positive for *C. burnetii*, including 5 cow, 2 sheep, and 2 goat milk samples (12). Other studies reported a different range of the presence of *C. burnetii* in milk. About 1.8% of the total goat milk samples and 0% of sheep milk in Iran were tested positive (9) whereas, in Japan, only 17.1% of the cheese samples were reported to be positive (13). The occurrence of food-borne microbial diseases in both humans and animals is increasing in developing countries due to poor health. On the other hand, developed countries indicated almost no cases of infections or food poisoning due to their high standards for health. As a result, the determination of the detailed statistics of the strike rate, especially for the developing countries, is impossible. The food itself can act as a carrier of the infection. For instance, in cases where the infection was contracted from non-plant based food in which most of the infectious and non-infectious bodies, which (under some conditions) supported an infectious mass and acted either as an active or only a passive carrier, demonstrated that the infection could only be transmitted through food. The examples of such food items are milk and dairy products. Furthermore, nowadays, with the increased knowledge of the consumption of milk and dairy products, it is important to have healthy milk and dairy products, especially for the elderly and infants who

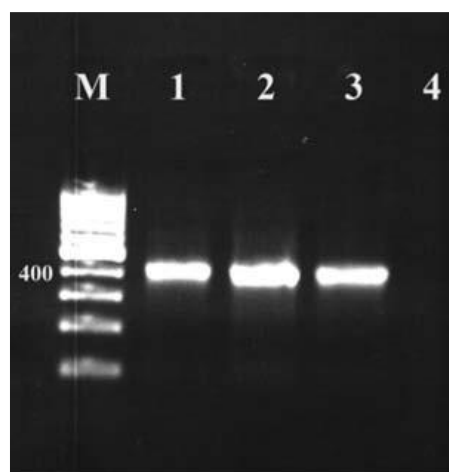


Figure 1. Nested Polymerase Chain Reaction *Coxiella burnetii* Samples of Traditional and Industrial Cream and Butter in Shahrekord. Note. Column M: 100 bp DNA marker; Columns 1 and 2: Positive samples of *C. burnetii*; Column 3: Positive control; Column 4: Negative control.

have weaker immunity compared to other individuals (14). The available nutrients (e.g., calcium, protein, and vitamins A-D) in dairy products are essential for strong bones and teeth and they reduce the risk of osteoporosis, help maintain a healthy nervous system, activate muscle contractions, and promote skin and eye health. Although Q fever is primarily an occupational disease, the consumption of contaminated milk and dairy products can play an important role in the epidemiology of the disease in humans (14).

Rodolakis proposed a hypothesis that suggested that the consumption of dairy products from *C. burnetii*-contaminated animals can lead to its food-borne infection in humans (15). In the research by Huebner and Jellison, the pasteurization of dairy products could remove *C. burnetii* from contaminated milk (16). Based on the results of another study, the highest prevalence of serum and clinical diseases was observed in patients who consumed raw milk (17). However, the results of these studies showed that most of the people living in rural areas eat raw milk and are exposed to ruminating mammals, indicating possible contamination from aerosol inhalation. Nevertheless, an acute fever outbreak in Newfoundland, Canada, was found to occur by eating pasteurized goat cheese as an independent risk factor (18). Moreover, in a study conducted in a prison, *C. burnetii* serology revealed a higher risk in those consuming raw milk (19). Conversely, no repression or clinical illness was reported in the study by Krumbiegel and Wisniewski, which included 34 volunteers who drank raw milk (20). *C. burnetii* DNA was detected in 64% of the dairy products in France (17), especially in dairy cows with a higher percentage. Nonetheless, no live bacteria were removed from cheese and yogurt in the mentioned study. Consequently, the digestive pathway may play an

important role in *C. burnetii* transmission even if it is not a major public health threat. The range of Q fever animal hosts is large and the disease spreads from ticks to primates. These microorganisms are found in the bodies of mollusks, arthropods, fish, birds, reptiles, marsupials, and livestock in many parts of the world (21).

Additionally, Nasehfar et al (22) found that clinically healthy dairy cows were the major sources of *C. burnetii* infection in Iran. In the current study, the milk samples of bovine bulk from central Iran were used to study the prevalence of *C. burnetii*. In the present study, 5 out of 100 (0.05%) bovine milk samples were tested positive for *C. burnetii*.

Q fever was first described by Derrick et al following the outbreak of flu-like illness among the slaughterhouse workers in Australia in 1937. It was considered as a zoonotic disease between livestock and humans, the spread of which was first reported in animals and then humans in Iran in 1952. The report of this disease in animals displayed evidence of a serological outbreak of fever while it was reported in humans in two patients showing severe fever and neurological symptoms associated with Q fever. In addition, this fever was first reported in Abadan, Khuzestan province, Iran. The unpasteurized samples of milk and dairy products in the study of Khanzadi et al (23) conducted in Mashhad, in Khorasan-Razavi province of Iran demonstrated positive touch-down PCR tests for *C. burnetii* in 2/28 cheese samples (7.14%), as well as 2/26 yoghurt samples (7.69%), 8/23 sheep milk samples (34.78%), and 2/60 bovine milk samples (3.33%).

Likewise, Kargar et al indicated that the prevalence of *C. burnetii* contamination in Jahrom in southern Iran was 11% after evaluating 100 bovine bulk milk samples (24). In other cities such as Fars, Qom, Kerman, Khuzestan, and Yazd, Abbasi et al, using a nested PCR assay, reported 2% goat milk samples (6/296), 18.2% samples from the dairy herds of Fars (4/22), and 4.2% (1/24) samples from the product herds of Khuzestan. In addition, the results showed product herds of Yazd as 5.5% (1/18), the 6 positive samples for the presence of *C. burnetii* (25).

Coxiella burnetii can survive for at least 586 days in the dried feces of ticks, and 3 months on the moist soil corrals of birthing sheep (1). Various factors can cause a relatively high prevalence of *coxiellosis* in cows. In the study by Dhaka et al (26), its prevalence was discovered to be 29.91%. According to the researchers in this field, the contributing factors to *coxiellosis* prevalence were as follows:

- Large herd size;
- Frieswal herds since older animals are more likely to become infected after their first deliveries;
- Inappropriate disposal of environmental wastes such as aborted materials and fertilizer on the farm;
- Low bio-security measures that allow stray dogs and wild birds enter the farm;

- High prevalence of reproductive disorders and other immune infections such as tuberculosis, brucellosis, Johne's disease, and mastitis (26).

In ewes, *C. burnetii* secretion often occurs in the vagina and feces while secretion milk can be the source of most bacteria in cows and goats (27).

The results of this study and those of other studies indicated that traditional dairy products could be considered as an important reservoir of *C. burnetii* infection in Iran. *C. burnetii* is a dangerous bacterium with unique features resulting in the bio-safety rating of level 3. Hence, only experienced people are permitted to work with the infected samples of *C. burnetii* to study its culture and isolation in laboratories. Further, *C. burnetii* is an obligate intracellular bacterium whose culture needs a live host, which is time-consuming and costly and involves high risk. Despite the suitability of serological tests to recognize its presence, it takes several weeks to produce the antibodies to fight against *C. burnetii*. Fortunately, this problem is limited to the diagnosis of acute Q fever. All the mentioned factors become even more important for the early diagnosis of *C. burnetii* through the standard molecular biology techniques. Therefore, it is recommended to establish standard setups for the diagnostic molecular approaches which are suitable to be used in our country (1).

Conclusions

The results of this study showed that *C. burnetii* was absent in the industrial butter and cream samples and thus there was no danger to public health, thanks to the management methods of food safety systems such as Hazard Analysis and Critical Control Point, Good Manufacturing Practice, and Good Health Practice employed in the dairy factories in Iran. In this research, the results of the nested PCR tests on the traditional butter and cream samples revealed that the samples were contaminated with *C. burnetii*. The findings of this study and those of other studies conducted in Iran in recent years have shown that Q fever is present in Iran while it is not considered to be an important issue or is confused with other febrile illnesses such as brucellosis or influenza due to the existence of few available studies in this field. Given the nature of zoonosis and its possible contamination through a host animal, the veterinary authorities and ministry of health subsectors should necessarily participate in the extensive and proximity interactions to identify its reservoirs and prevent or control the disease. Regarding its health and economic importance in humans and livestock, it is suggested to identify the disease repository of ruminants after slaughter and to vaccinate those people at risk, including veterinarians and slaughterhouse workers.

Conflict of Interests

None.

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