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Prevalence of *Coxiella burnetii* in unpasteurized dairy products in west of Iran

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ABSTRACT

Q fever is a zoonotic disease caused by *Coxiella burnetii*. This study investigated the prevalence of *C. burnetii* in traditional dairy products, specifically yogurt and cheese, in Lorestan Province. A total of 100 samples of traditional yogurt and unpasteurized cheese were collected from various regions. To analyze the genome of *C. burnetii*, DNA was purified and molecular detection was performed using nested PCR with primers specific to the *IS1111* transposon gene. The results revealed a prevalence of 13.3 % (95 % CI: 6.9 %–24.16 %) in yogurt samples and 12.5 % (95 % CI: 5.46 %–26.11 %) in cheese samples. Additionally, a significant seasonal variation in contamination levels was observed, with a *p*-value of less than 0.05. However, no significant correlation was found between geographical location and the degree of contamination. These findings suggest that the contamination of dairy products with *C. burnetii* is likely due to the bacterium's 'spore-like' form and the lack of pasteurization in the traditional production of yogurt and cheese. While the direct risk of transmission via unpasteurized dairy products is considered low, these products should still be monitored in Q fever outbreaks.

1. Introduction

Coxiella burnetii is a minute obligate intracellular bacterium with a membrane structure analogous to that of other gram-negative bacteria (Eldin et al., 2017; Van Schaik et al., 2013). Previously, *C. burnetii* was classified within the order *Rickettsiales*. However, more recent phylogenetic research based on *16S rRNA* sequence analysis has led to its reclassification as a member of the class Gammaproteobacteria, order Legionellales, and family Coxiellaceae (Frankel et al., 2011; Wentworth, 1955). *C. burnetii* is a zoonotic and highly infectious bacterium that can be transmitted from animals to humans, with the potential to cause Q fever (Amin et al., 2022; Khademi et al., 2020). This bacterium has two forms, small and large. One of these cell types, characterized by dense chromatin, is believed to be an extracellular survival form, which demonstrates remarkable resistance to environmental stressors such as desiccation and heat (Asadi et al., 2013).

C. burnetii is a major pathogen in livestock, including cattle, sheep, and goats, and it is found in urine, feces, milk, and birth fluids of infected animals. The primary risk factors for this pathogen include its presence in livestock, the possibility of survival during milk processing, and its transmission through inhalation and ingestion (Gale et al., 2015). traditional methods for identifying and

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enumerating it have often failed (Rozental et al., 2020). The pathogen is primarily spread through infected aerosols, but consumption of unpasteurized milk and cheese is considered less of a risk factor for human infection. While *C. burnetii* is recognized as a major tick-borne pathogen, its role in human infections remains unclear (Mege et al., 1997; Porter et al., 2011).

Since the 2007 epidemic in the Netherlands, there has been increased global interest in studying Q fever (Mostafavi et al., 2019; Schneeberger et al., 2014). Despite some unreliability and lack of data, evidence from developed countries indicates that raw milk consumption may lead to Q fever. Recent reports have identified *C. burnetii* in Michigan, USA, in five cases (Rozental et al., 2020; Signs et al., 2012).

However, there is limited evidence that processes involved in the manufacture of non-pasteurized cheese, cream, or butter effectively inactivate *C. burnetii*. Huebner and Jellison (1949) reported that infective *C. burnetii* persisted in butter made from infected milk, and could survive in milk dried at 37 °C for 30 to 60 days, and in cheese made from infected milk for 17 to 46 days (Badudieri and Moscovici, 1950). Zubkova (1957) found that *C. burnetii* could survive in sterile milk at room temperature for up to 125 days (Zubkova, 1957). Sipka (1958) detected viable pathogens in cottage cheese after 42 days (Sipka, 1958), though inactivation rates could not be precisely determined due to the lack of quantitative data. Studies of unpasteurized small ruminant cheeses have shown that 32 % were PCR-positive, with nearly two-thirds being non-industrialized cheeses.

The *IS1111*-insertion sequence, which encodes a transposase, is present in up to 56 copies in the genomes of *C. burnetii*. Consequently, this element is frequently employed as a specific target for the development of sensitive diagnostic PCRs (Khademi et al., 2024).

It is supposed that the inner inverted repeats act as the endpoints of the *IS1111* element which forms a circular intermediate causing the inner inverted repeats to come into closer proximity and create a promoter enhancing transposase expression. Conversely, the outer inverted repeats likely act as chromosomal sequences that create a stem-loop structure functioning as a site for the insertion of the *IS1111*. This specific target sequence appears over 50 times in the 9Mi/I genome (Khademi et al., 2024; Partridge and Hall, 2003).

The use of polymerase chain reaction (PCR) with specific primers targeting the pathogen's transposon-like element sequence (*IS1111*) has enhanced the sensitivity of *C. burnetii* detection (Berri et al., 2000; Berri et al., 2002; Boldis et al., 2012; Khademi et al., 2020; Khademi et al., 2024; Parisi et al., 2006; Partridge and Hall, 2003). The present study was conducted in Lorestan Province, located in western Iran, with the objective of identifying the genomic of *C. burnetii* in traditional cheese and yogurt samples through the use of nested-PCR methodology, based on primers that are specific to the *IS1111* transposon gene.

2. Materials and methods

2.1. Sample collection

A total of 400 traditional cheese ($n = 200$) and yogurt (n = 200) samples were randomly collected from fifty traditional dairy shops in four different geographical regions of Lorestan (north, center, south, and west) during the spring and summer of 2022. The collected cheese and yogurt samples were placed on ice and immediately transferred to the microbiological laboratory at the Vet School (Fig. 1).

2.2. DNA extraction from milk samples

The procedure outlined by Khademi et al. (2020) was used to prepare cheese and yogurt samples for DNA extraction. First, the cheese samples were divided into smaller portions of 50.00 g each and packaged in sterile bags (Saniplast Mehr, Tehran, Iran) for solid or liquid samples. The bags were sealed, identified, macerated, and homogenized. The samples were centrifuged at 14,000 rpm for 10

Fig. 1. The schematic map of the study areas, Lorestan, Iran.

min. The fat and supernatants were then discarded, and the 50 g remaining pellets were washed with sterile PBS. Next, the pellets were re-suspended in 100 μ*l* of distilled water and used for DNA extraction. DNA was extracted using a Blood Genome DNA Mini Kit (50 samples) following the manufacturer's instructions (Cat No: FABGK001, Favorgen, Taiwan). The quality and quantity of the extracted DNA were assessed with a NanoDrop 2000c (Thermo Scientific, USA), and the DNA samples were stored at −20 °C until needed for PCR.

It is crucial to acknowledge the potential impact of milk and dairy products on the efficacy of PCR-based assays. The presence of fat and protein in such products has the potential to impede the precision of these assays (Baptista et al., 2021; Jana et al., 2020).

Moreover, the utilization of spin columns has been demonstrated to be an efficacious approach for the isolation of superior-quality DNA from milk and dairy products, while concurrently reducing the concentration of inhibitory substances (Baptista et al., 2021).

2.3. Nested PCR for molecular detection of C. burnetii

Nested PCR targeting the *IS1111* transposon gene was employed for the molecular detection of *C. burnetii*. The primers used for this nested PCR were previously described by Parisi et al. (2006) and Berri et al. (2000) (see Table 1).

In one study, the *com*1 gene region was employed, while another study utilized a combination of *com*1, *htp*—B, and *icd* gene regions. Studies employing the *IS1111* gene region revealed a proportion of positive samples relative to the total number of cheese samples of 29.2 %. However, this rate was reduced to 15.5 % in two additional studies conducted in Iran and Japan. It has been demonstrated that the *IS1111* gene region displays varying copy numbers contingent upon the specific strain, thus conferring enhanced sensitivity (Khademi et al., 2024; Rabaza et al., 2021).

For the first stage of nested PCR, Taq DNA Polymerase Master Mix RED (No.: A190303 Amplicon, Denmark) was used. The PCR reaction was set up in a 25 μ*l* volume, including 5 μl of extracted DNA, 50 picomoles of each primer (Trans 1 & Trans 2), and 12.5 μl of master mix. The PCR conditions were optimized to minimize contamination and inhibition, while enhancing specificity and sensitivity. The thermal cycling conditions for both touchdown and trans-PCR were configured using a thermal cycler (Quanta Biotech, UK) as previously described. For the nested PCR stage, the reaction mixture was prepared similarly, but with 2.5 μl of a 1:100 dilution of the PCR product from the first stage as the DNA template. The thermal cycling conditions followed those outlined by Khademi et al. (2020). PCR products from both stages were analyzed using electrophoresis on a 2 % agarose gel containing Safe Stain and visualized with Ingenious Gel Documentation (Syngene Bio Imaging, UK) (see Fig. 2).

2.4. Statistical analysis

Statistical analysis was performed using the chi-square test with SPSS software (version 22.0; IBM Corporation). The threshold of statistical significance was considered to be a *p*-value of less than 0.05.

3. Result

3.1. Touchdown NESTED PCR test results in traditional unpasteurized yogurt and cheese

Eight out of 60 yogurt samples (13.3 %, CI: 6.9 %-24.16) and five out of 40 cheese samples (12.5 %, CI: 5.46 %–26.11 %) were positive for the presence of *C. burnetii*. The frequency distribution of *C. burnetii* contamination of local yogurt and cheeses according to geographical region and season is shown in Table 2.

3.2. Traditional yogurt and cheese contamination with C. burnetii according to season

Yogurt samples collected in winter showed the highest level of infection, four out of 15 (26.6 %), while samples collected in spring and summer showed the lowest level of infection, one out of 15 (6.6 %), and samples collected in autumn showed the lowest level of infection, two out of 15 (3/13 %). Similarly, the highest level of contamination of traditional cheeses with *C. burnetii* was observed in winter, 3 out of 10 samples collected (30 %). One out of 10 samples collected in autumn (10 %) and one out of 10 samples collected in spring (10 %) were positive, while no positive samples were recorded in summer. The seasonal effect on the frequency of *C. burnetii* infection was demonstrated by a statistically significant difference ($p < 0.05$) of *C. burnetii* contamination in traditional yogurt and cheese samples across different seasons (Table 2).

Table 1
Primer sequences for detection of ***C. burnetii*** IS1111 gene by nested PCR (Parisi et al., 2006).

Protocol	Primer Name	Sequence 5′——3’	PCR product size (bp)
Trans-PCR	Trans 1	TATGTATCCACCGTAGCCAGTC	687
	Trans 2	CCCAACAACACCTCCTTATTC	
Nested-PCR	261F	GAGCGAACCATTGGTATCG	203
	463R	CTTTAACAGCGCTTGAACGT	

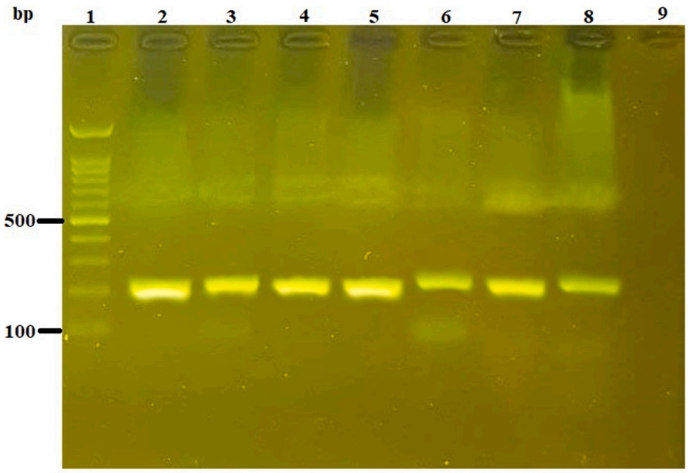

Fig. 2. Agarose gel image of amplified fragment of *C. burnetii IS1111* gene (203 bp) using nested-PCR. Lane 1, 100-bp molecular ladder (Smobio Technology Inc., Taiwan); Lane 2, Positive control (Nine Mile strain), lanes 3–8 positive samples for *C. burnetii,* lane 9, negative control.

Table 2
Frequency distribution of local yogurt and cheese contamination with *C. burnetii* according to geographic region and season.

Dairy product	category	(positive %) and the number of cheese samples	(positive %) and the number of our yogurt samples	The total sum (positive percentage) and the total number of samples
Township	*Alashtar*	(1; 12.5) 8	(1; 10) 10	(2; 11.1) 18
	Borujerd	(2; 25) 8	(1; 10) 10	(3; 16.6) 18
	Khorramabad	(2; 25) 8	(5; 25) 20	(7; 25) 28
	Chegani	(0) 8	(1; 10) 10	(1; 10) 18
	Pol-e-Dokhtar	(0) 8	(0; 0) 10	(0; 0) 18
Season	Spring	(1; 10) 10	(1; 6.6) 15	(2; 8) 25
	Summer	(0) 10	(1; 6.6) 15	(1; 4) 25
	Autumn	(1; 10) 10	(2; 13.3) 15	(3; 12) 25
	winter	(3; 30) 10	(4; 26.6) 15	(7; 28) 25

3.3. The results of contamination of traditional yogurt with C. burnetii based on geographical location

The highest level of contamination was observed in the central region of Lorestan Province (Khorramabad), with 25 % of yogurt samples (5 out of 20) and 25 % of cheese samples (2 out of 8) testing positive for *C. burnetii*. In Chegani, 10 % of yogurt samples (1 out of 10) were positive, while none of the cheese samples (8 total) were contaminated. In Al-Shatar, 10 % of yogurt samples (1 out of 10) and 12.5 % of cheese samples (1 out of 8) were positive for *C. burnetii*. In Borujerd, 10 % of yogurt samples (1 out of 10) and 25 % of cheese samples (2 out of 8) were found to be infected. In contrast, no contamination was detected in the 18 samples of yogurt and cheese collected from Pol-e-Dokhtar. This analysis showed the highest contamination rates in Khorramabad and the lowest in Pol-e-Dokhtar. However, no statistically significant differences in contamination levels were found among the various study areas, suggesting that geographic location does not significantly influence the level of *C. burnetii* contamination in dairy products (see Table 2).

4. Discussion

Q fever is one of the most frequently investigated serologically and molecularly serological and molecular investigated animal diseases (Esmaeili et al., 2014; Esmaeili et al., 2019; Khademi et al., 2014; Khademi et al., 2020; Khademi et al., 2019; Muskens et al., 2011). The important role of ruminants in this disease is well-known. However, during an epidemic in Newfoundland, Canada, consuming pasteurized goat cheese was identified as an independent risk factor for acute Q fever. Children who reported consumption of cheese from rural areas in Greece and goat workers who ate cheese made from pasteurized goat milk in Newfoundland had increased risk for Q fever.

Numerous studies have detected *C. burnetii* DNA in milk and derived products, including cheese, cream, butter and yogurt from cows, goats and sheep (Pearson et al., 2014). The current study findings demonstrated that 13.3 % and 12.5 % of yogurt and cheese samples were infected with *C. burnetii,* respectively.

A molecular study of the most traditional and oldest type of raw-milk cheese in Brazil, known as Minas, a cow's milk artisan cheese, showed that *C. burnetii* was prevalent and that an estimated 1.62 tons of cheese made daily was contaminated (Pearson et al., 2014).

In addition, in the United States, *C. burnetii* has also been isolated from unpasteurized bovine milk. Molecular studies indicate that

the dominant *C. burnetii* genotypes in dairy products are the same as those infecting dairy cows (Tilburg et al., 2012). Traditional cheeses and yoghurts made from cows in the Lorestan province of western Iran were used in this study.

Some studies have reported the presence of *C. burnetii* DNA by PCR in unpasteurized cheeses. For example, in the study by Capuano et al. (2012), 3.21 % of cheeses produced with unpasteurized milk in southern Italy were infected with *C. burnetii* (Capuano et al., 2012). Similarly, in the study by Hirai et al. (2012), out of 41 commercial cheese samples made from unpasteurized milk, seven cheeses were infected with *C. burnetii* using the PCR method (Hirai et al., 2012). A meta-analysis study was conducted using the mean and standard deviation values obtained from 13 original studies (Yanmaz and Ozgen, 2024). The overall molecular prevalence of *C. burnetii* in cheese was estimated to be 25.2 % (95 % confidence interval [CI]: 13.1 %–39.7 %).

In a serological survey of a cohort of goatherders, goat workers and their contacts involved in the Newfoundland outbreak in Canada, the use of pasteurized goat milk cheese emerged as a significant independent risk factor for infection. Similarly, in a two-year epidemiological study of 1200 Greek children admitted to hospital, the consumption of raw cheese from rural areas was associated with an increased risk of Q fever ($p = 0.04$, odds ratio:6, and 95 % CI:1.10–33.20 %) (Maltezou et al., 2004).

A 2007 Swedish study found that out of 359 cow's milk samples collected from cheese dairies, 17 samples, or 7.40 %, contained (Maltezou et al., 2004). *C. burnetii* was found in 3.50 % of 400 milk samples from 23 sheep flocks in a similar study conducted in Turkey. Nine out of 21 milk samples were reported positive for *C. burnetii* in another study conducted in the United States in 2010 (Loftis et al., 2010). Prevalence of 0.00 %–48.00 % in milk samples has been reported in studies from different geographical areas of Iran.

For example, the contamination rates in central (Chaharmahal and Bakhtiari provinces), southern (Jahrom), western Azerbaijan, northwestern (Bonab) and southwestern (Khuzestan province) regions of Iran have demonstrated prevalence of 6.20, 11.00, 16.90, 26.00 and 1.10 %, respectively (Nokhodian et al., 2017).

The most recent study by Makarizadeh et al. (2020) reported a contamination rate of 50.12 % for Kope cheese using the nested PCR method (Mokarizadeh et al., 2023), while the study by Khademi et al. (2020) reported a contamination rate of 7.60 % for sheep's milk and 16.60 % for goat's milk. Differences in the prevalence of *C. burnetii* in dairy products around the world are due to differences in the climate and environment of geographical areas, the type of survey, the type and number of samples and the season of sampling (Kim et al., 2005; Rodolakis et al., 2007; Tilburg et al., 2012).

One satisfactory explanation for *C. burnetii*'s ability to form spore-like environments is that it is resistant to colonization. This has been recognized as a factor in the long-term survival of the bacterium in milk and dairy products. Results from unpasteurized cheese showed low viability of *C. burnetii*, in contrast to unpasteurized milk. A 2013 study found that *C. burnetii* was not viable in PCR-positive dairy products (Eldin et al., 2013), and that there was no risk to consumers. Detection of *C. burnetii* viability in hard cheese after eight months of maturation would be a worst case (Barandika et al., 2019), but few studies have gone further to examine viability and risk. Culture in Vero cells and inoculation in mice have demonstrated viable *C. burnetii* in raw cheese (Barandika et al., 2019).

There have been differences of opinion on the use of heat treatment of the milk in the production of cheese. For these reasons, it is advisable to lay down minimum requirements for farmers whose milk is used to make these types of cheeses. In the following areas, the identification of positive individuals or flocks by serological or PCR testing, herd confinement, health certification requirements when animals are purchased, vaccination, prenatal pre-natal separation and removal of placenta and fetal complications are some of the recommended procedures to avoid any complications (Rozental et al., 2020).

The relatively low level of contamination observed in some areas, such as Pol-e-Dokhtar, may be attributed to the implementation of effective sanitary practices on local livestock farms. The estimated overall molecular prevalence of *C. burnetii* in cheese was found to be 25.2 % (95 % CI 13.1 %–39.7 %). These findings align with previous studies that have identified the presence of *C. burnetii* in cheese samples from various geographical regions (Basanisi et al., 2022; Eldin et al., 2013; Gyuranecz et al., 2012). The considerable heterogeneity observed across studies, with an I2 value of 96.3 %, underscores the need for cautious interpretation of the data. Variations in prevalence estimates among different regions and types of cheese can be attributed to differences in sampling methodologies, the sample size, and the sensitivity and specificity of molecular techniques utilized for diagnosis.

Moreover, variations in the prevalence of *C. burnetii* in animal populations may also contribute to the observed heterogeneity (Pearson et al., 2014). Despite the high degree of heterogeneity, the absence of significant evidence of publication bias, as indicated by Egger's test, adds credibility and reliability to the results.

The discrepancy in the prevalence of *C. burnetii* in dairy products across different geographical regions can be attributed to a number of factors, including variations in climate and environmental conditions, the methodology employed in the surveys, the type and number of samples collected, and the timing of sampling. These factors have been identified as key contributors to the observed differences in the prevalence of *C. burnetii* in dairy products across diverse geographical regions (Khademi et al., 2019; Mazeri et al., 2013).

The cheeses subjected to analysis in this study were obtained from establishments after a period of 2–3 days on the market, a timeframe that may potentially facilitate the transmission of bacteria to consumers of such products. The cheese production chain is vulnerable to contamination by *C. burnetii*, which is thought to have a high resistance capacity, allowing it to persist and be detected at the end of the production process (Angelakis and Raoult, 2010). Contamination can be caused by the raw material, which may originate from cattle infected with the bacterium, or by the manufacturing and storage of the finished product.

The most significant limitation of this study is that the cheeses were procured at the conclusion of the production process, which precludes the ability to determine the precise point of contamination by the Q fever agent. Additional constraints were imposed by the lack of collaboration from cheese producers and retailers. Additionally, the limited sample size presented challenges in data analysis and the calculation of confidence intervals.

5. Conclusion

This study is the first to examine the prevalence of *C. burnetii*, the bacterium responsible for Q fever, in cheese and yogurt from the Lorestan region. The findings suggest that local cheese and yogurt may significantly impact the epidemiology of *C. burnetii* in western Iran. The high prevalence of *C. burnetii* in unpasteurized dairy products confirms that these items are important reservoirs and sources of the bacterium, potentially making them major contributors to human contamination in this region. Therefore, there is a clear need for additional research to assess contamination levels across different areas of the country, as well as for implementing effective control measures and timely interventions to prevent the spread of the disease. The findings confirm that these dairy products serve as reservoirs and sources of the bacterium. In summary, it is advised that pasteurization techniques, particularly the high-temperature, short time (HTST) method, be employed to minimize the presence of *C. burnetii* in milk and fluid milk products.

CRediT authorship contribution statement

Soheila Mohammadkhanifard: Methodology, Formal analysis. **Amin Jaydari:** Writing – review & editing. **Ehsan Rashidian:** Writing – review & editing. **Nemat Shams:** Writing – review & editing. **Peyman Khademi:** Writing – review & editing, Writing – original draft, Software, Methodology.

Declaration of competing interest

Authors declare there is no conflict of interests.

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