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Prevalence of *Coxiella burnetii* in milk collected from buffalo (water buffalo) and cattle dairy farms in Northwest of Iran

Payman Khademi[a], Abdulghaffar Ownagh[a,*], Karim Mardani[b], Mohammad Khalili[c]

[a] Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, West Azerbaijan, Iran
[b] Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, West Azerbaijan, Iran
[c] Department of Pathobiology, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran

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ABSTRACT

The present study was conducted to determine the prevalence of *C. burnetii* in raw milk samples collected from water buffalos and cattle in Northwest of Iran (West Azerbaijan Province). A total number of 840 milk samples were randomly collected from buffalos and cattle belonged to three different geographical regions in west Azerbaijan (the map is necessary). The milk samples were collected seasonally during 2018 and the age of animals were recorded. All the milk samples were subjected to DNA extraction. Nested-PCR was used to detect *C. burnetii* based on the transposable gene *IS1111*. The results showed that 16.9% (95% CI: 14.5%–19.6%) of the examined milk samples (19.3% buffalo and 14.6% cattle samples) were positive for *C. burnetii*. There was a significant difference in *C. burnetii* shedding in milk between different age groups in cattle but not in buffalos (p value < 0.05). The shedding of *C. burnetii* in milk was highly prevalent in summer (31.1%) ($p < 0.05$, 95% CI: 26.1%–38.4%). There were significant regional and seasonal variations in the prevalence of *C. burnetii* in the examined milk samples. It was concluded that buffalo population in west Azerbaijan should be considered as an important factor in the epidemiology of Q fever and consequently in public health.

1. Introduction

Coxiella burnetti as a global zoonotic agent, is responsible for Q fever disease in human and animals [1,2]. It is a Gram-negative intracellular bacterium distributed across the globe except in New Zealand and Antarctica. Farm animals such as cattle, sheep, goats, and cats are the main sources of Q fever infection in human [3–6]. The transmission of *C. burrnetti* from animals to human is typically through respiratory route and the urine and feces of infected animals [3,7,8]. The contamination of raw milk with *C. burnetii* raises concern over the role of milk as a source of infection in human. Recent studies from different countries have demonstrated that the prevalence of *C. burnetii* in raw milk can vary over a wide range from 4.7% to as high as 47.7% [9–17]. Therefore, based on the recent reports, the risk of *C. burnetii* infection by consuming unpasteurized milk products cannot be considered negligible.

Although immunofluorescence, enzyme-linked immunosorbent assay (ELISA) and complement fixation (CF) tests are the most commonly used methods for diagnosing Q fever, these methods have major drawbacks for detection of Q fever agent [8,18,19]. On the other hand, the isolation of *C. burnetti* is not recommended as a common diagnosis method in veterinary medicine due to its difficulty, time-consuming and it requires a confined level three laboratory [20].

Large ruminants, especially domestic water buffalo (Bubalus bubalis) is an important livestock species worldwide as it is a source of high quality milk, meat and leather. The contact between water buffaloes and other domestic and wild animals, especially cattle, as well as their access to different ecosystems, has exposed this species to different infectious diseases [21]. With the population of 160,000 buffalos and 800,000 cows, the meat and dairy products of these two domestic animals are the main sources of income in the rural areas of west Azerbaijan [22].

Milk is a major source for many pathogens, especially pathogenic bacteria. The role of milk as an important vector of infectious pathogens has been identified since the past decades with various epidemic insurgence such as *S. aureus*, *Mycobacterium bovis*, *C. burnetii* and Salmonella spp. [23]. The milk of infected livestock is an important way to excrete *C. burnetii* into the environment. Therefore, the consumption of non-pasteurized and contaminated milk is one of the main transmission methods of *C. burnetii* to human [24].

Many authors considered the polymerase chain reaction (PCR) to be an innovative procedure for *C. burnetti* detection in the biological

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[*] Corresponding author.
E-mail address: a.ownagh@urmia.ac.ir (A. Ownagh).

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samples [25–28]. According to the previous literatures, the PCR method is a well-established laboratory procedure for detection of *C. burnetti*. In addition, this method is capable to detect particular DNA sequences in a low number in the biological samples. A PCR using specific primers targeting transposon-like element sequence (IS1111) of the agent has been demonstrated to be especially a sensitive method for detection of *C. burnetti* [29].

The current study was planned to investigate the prevalence of *C. burnetii* in buffalo (for the first time in Iran) and cattle from dairy farms of West Azerbaijan Province, Iran.

2. Materials and methods

2.1. Study areas

This study was carried out in West Azerbaijan Province located in the northwest of Iran, geographically located between 37° 33′ 10.08″ N, 45° 4′ 33.24″ E (https://tools.wmflabs.org). The climate of west Azerbaijan is largely influenced by the rainy winds of the Atlantic Ocean and Mediterranean. Cold northern winds affect the province during winter and cause heavy snow (https://www.britannica.com/place/Azerbaijan-region-Iran) (Fig. 1).

2.2. Milk sampling

A number of 840 milk samples were randomly collected from buffalos (n = 420) and cattle (n = 420) belonged to 84 dairy farms in three different geographical regions of west Azerbaijan (north, center and south) during four seasons in 2017. Sampled animals were grouped into three different age groups (0–6, 7–10 and over 10 years old). The collected milk samples were placed on ice and immediately transferred to the microbiology laboratory at the Faculty of Veterinary Medicine.

2.3. DNA extraction from milk samples

Milk samples were prepared for DNA extraction according to the procedure described by Berri et al. [30]. Briefly, the sample was centrifuged at 14,000 rpm for 10 min. The fat and the supernatant were discarded and the resultant pellet washed using sterile PBS. The pellet was re-suspended in 100 µl distilled water and was used for DNA extraction. DNA extraction from was carried out Blood Genomic DNA Extraction Mini Kit (50 preps), (Favorgen, Taiwan) according to the kit's manufacturer instructions. The quality and amount of extracted DNA was evaluated by Nano Drop 2000c (Termo Scientific, USA). Extracted DNA samples were kept at −20 °C for the later use in PCR.

Table 1
Primer sequences for detection of *C. burnetii* IS1111 gene by nested PCR.

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	

2.4. Nested-PCR for the molecular detection of C. burnetii

For the molecular detection of *C. burnetii* nested-PCR targeting the transposon IS1111 gene was employed. The primers for the trans-PCR and nested-PCR which were used in this study previously described by Parisi et al. [5] and Berri et al. [29] respectively (Table 1).

To perform the first stage of the nested-PCR, Taq DNA Polymerase Master Mix RED (Amplicon, Denmark) was used. The PCR reaction was carried out in 25 µl volume comprising 5 µl of extracted DNA, 50 pmol of each primer (Trans 1 & Trans 2), 12.5 µl of mater mix. The touchdown PCR was used to optimize and reduce contamination and inhibitors, as well as increasing the specificity and sensitivity of the reaction. The touchdown and Trans-PCR thermal programs were defined in the thermal cycler device (Quanta Biotech, England) as described previously [29].

For the nested-PCR stage, PCR reaction was prepared as the Trans-PCR which described previously except for the DNA template, which 2.5 µl of 1:100 diluted PCR product from the first stage was used. The thermal cycling condition was described according to Parisi et al. [5]. The PCR products for both stages were electrophoresed on a 2% agarose gel containing safe stain and then visualized using Ingenius Gel Documentation (Syngene Bio Imaging, UK) (Fig. 2).

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

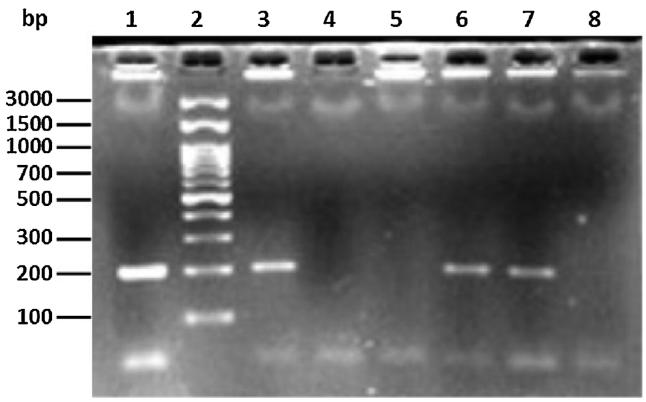

Fig. 2. Agarose gel image of amplified fragment of *C. burnetii* IS1111 gene (203 bp) using nested-PCR. Lane 1, Positive control (Nine Mile strain), Lane 2, 100-bp molecular ladder (Smobio Technology Inc., Taiwan); lanes 3, 6, 7 positive samples for *C. burnetii*, Lane 4, 5, negative samples for *C. burnetii,* lane 8, negative control.

2.5. Data analysis

The obtained data were statistically analyzed by Chi-square test using SPSS software Ver. 22 (SPSS Inc., Chicago, IL). The P value < 0.05 was considered significant.

3. Results

3.1. Nested-PCR amplification of IS1111 gene

Among 840 collected milk samples, 142 samples (16.9%) were positive for *C. burnetii* amplifying a fragment of 203 bp of the IS1111 gene using nested-PCR. A number of 81 (19.3%) and 61 (14.6%) milk samples from buffalos and cattle were positive for *C. burnetii* respectively. The prevalence of *C. burnetii* in the milk of two examined species were not statistically significant. All the animals subjected to milk collection were classified based on their age into three age categories (0–6, 7–10 and over 10 years old). There was a significant difference in milk contamination with *C. burnetii* between the defined age groups in cattle ($P < 0.05$), in which there was an increase in the *C. burnetii* shedding in cattle milk with the increase in their age. However, in spite of the same pattern of the increase of the bacterium shedding in buffalo milk with the increase of the age, the difference between different age groups of buffalo in terms of bacterium shedding in milk was not significant (Table 2).

Table 2
Prevalence of *C. burnetii* in the milk samples collected from buffalo and cattle dairy farms in different seasons and regions of West Azerbaijan Province.

Animal			Total
	Buffalo	Cattle	
Age group (Years old)			
< 6	9/106 (8.5%)	11/100 (11%)	20/206 (9.7%)
7-10	44/273 (16.1%)	59/277 (21.3)	103/550 (18.7%)
> 10	8/41 (19.5%)	11/43 (25.5%)	19/84 (22.6%)
Season			
Spring	7/104 (6.7%)	12/105 (11.4%)	19/209 (9.1%)
Summer	31/113 (27.4%)	39/106 (36.8%)	70/219 (31.1%)
Autumn	23/98 (23.5%)	30/104 (28.8%)	53/202 (26.2%)
Winter	0/105 (0.0%)	0/105 (0.0%)	0/210 (0.0%)
Region			
North	32/136 (23.5%)	43/139 (30.9%)	75/275 (27.3%)
Center	20/141 (14.2%)	26/140 (18.6%)	46/281 (16.4%)
South	9/143 (6.3%)	12/141 (8.5%)	21/284 (7.4%)

3.2. The seasonal and geographical distribution of contaminated milks with *C. burnetii*

The highest frequency of positive milk samples for *C. burnetii* was in summer (31.1%) ($P < 0.05$, 95% CI: 26.1%–38.4%), while no positive sample was detected in winter. The highest number of positive milk samples belonged to the south region of the west Azerbaijan. The prevalence of *C. burnetii* in the examined milk samples collected in different seasons and regions were significantly differed ($P < 0.05$) (Table 2).

4. Discussion

The present study was the first epidemiological study on Q fever in buffaloes and cattle dairy farms of West Azerbaijan province. Results of the present study revealed that 16.9% of the all examined raw milks were positive for *C. burnetii*. The prevalence of *C. burnetii* in buffalo milk (19.3%) was higher than cattle (14.6%). To the best of our knowledge, to date, there is only one report on the prevalence of *C. burnetii* in buffalo which it was in India. They reported that the overall *C. burnetii* prevalence in buffalo and cattle was 7.0% with 8.7% in cattle and 4.3% in buffalo [31]. These findings were not in agreement with our results which can be related to the geographical differences, type of the samples that was examined and the farms management practice.

There are many studies in Iran and the other countries reporting the prevalence of *C. burnetii* in cow's raw milk. In Khorasan-Razavi, East Azarbaijan, Tehran and Zanjan provinces the prevalence of *C. burnetii* in milk were 5.0%, 25.0%, 12.0% and 13.2% respectively [27,32–34]. In the other countries, the prevalence of *C. burnetii* in cattle milk was reported at the range of 4.7–53.7% with the lowest and highest prevalence reported from Switzerland and Japan respectively [9,11–16,35]. Based on the reported data, the prevalence of *C. burnetii* varied in different geographical areas. The reasons for these discrepancies in the prevalence of *C. burnetii* may be due to the farms' hygienic level, type of animal husbandry, geographical area and the climate. The higher prevalence of *C. burnetii* in cattle of west Azerbaijan (14.6%) in comparison with other provinces in Iran may be due to the presence of buffalos in West Azerbaijan which has been showed that there is an increased risk of a cattle becoming infected when in contact or sharing a common grazing area with buffalos [36].

The findings of the current study are in agreement with the similar studies from Iran and the other countries. Based on the prevalence findings reported by many researchers, *C. burnetii* is more prevalent in cow milk compared to other animal's milk. The reason for the high prevalence of *C. burnetii* in cattle milk in comparison with the other animals such as sheep has been related to the fact that in cattle vaginal shedding of *C. burnetii* is normally very short (< 14 days) while shedding in milk continues for much longer periods and also in sheep bacterium shed mainly via feces and vaginal mucus [37,38]. Therefore, cattle milk can play an important role in the epidemiology of Q fever and can have a major impact on public health.

In the present study it was revealed that there was a significant association between the age and *C. burnetii* shedding in cow's milk. This finding was in agreement with the previous report in which it was showed that age was a significant risk factor for shedding of *C. burnetii* in cattle milk and the chance for a positive result increased 1.67 times for an increase in each year age [39]. The results of the present study showed that there was a significant regional variation in the shedding of Q fever agent in raw milk. The highest contamination of milk with *C. burnetii* was in south of the province. It has been reported that the regional distribution of Q fever in human cases is similar to the distribution and density of sheep and cattle populations. Therefore, it might be speculated the population of buffalo, cattle and sheep shedding the bacterium will increase the positive samples [40].

In a study by van der Hoek et al. a seasonal pattern of the onset of Q fever in human in spring and early summer was reported [41]. It was also demonstrated that in animals the increased incidence of Q fever is linked to lambing season so that in many European countries, the highest numbers of cases were reported during summer due to spring lambing season [5,24,42]. In the present study the highest prevalence of *C. burnetii* shedding in milk was in summer which was in accordance with the previous report [5,24,42].

5. Conclusion

In conclusion the results of the present study indicated that the raw milk of buffalo and cattle can be important sources of Q fever agent. The age can be considered as an important risk factor in the prevalence of *C. burnetii* in raw milk. The shedding of *C. burnetii* in milk follows a seasonal and regional pattern. It was revealed that buffalo can play an important role in the epidemiology of Q fever in west Azerbaijan and should be taken into consideration in terms of public health.

Declaration of Competing Interest

Authors declare there is no conflict of interests.

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