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Molecular detection of *Coxiella burnetii* in Kope cheese and cattle milk in West Azerbaijan, Iran

Kosar Mokarizadeh[1], Addolghaffar Ownagh[2]*, Hossein Tajik[3]

[1] DVM Student, Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [3] Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

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Abstract

There are few studies on *Coxiella burnetii* (Cb) as a causative agent of Q fever in dairy products in Iran. The prevalence of Cb was studied by polymerase chain reaction (PCR) method in Kope (pot) cheese and cattle milk collected from West Azerbaijan province, Iran. A total number of 240 Kope cheese and 560 milk samples were collected during the year 2020. All samples were subjected to PCR based on transposable gene *IS1111*. The results showed that 12.50% (95.00% confidence interval (CI): 9.00 - 16.10%) of Kope cheese and 13.00% (95.00% CI: 10.00 - 17.30%) of milk samples were positive for Cb. There was a significant difference in cheese and milk contaminations with Cb among the defined age groups as well as regional and seasonal variations. It was concluded that Kope cheese and cattle milk are important sources of Cb and should be considered as important risk factors in the epidemiology of Q fever disease in public health.

Introduction

Coxiella burnetii (Cb) is an intra-cellular Gram-negative pathogenic bacterium, being a causative agent of Q fever.[1] Taken a whole, two cell types with morphologically distinctive feature including bi-phasic growth cycle are generated by a bacterium having a form of small Gram-negative pleomorphic coccobacilli. A small cell type with dense chromatin has been considered as a form of extra-cellular survival indicating a remarkable resistance feature to environmental stress factors such as desiccation and heat.[2]

The important issues of the risk profile include investigation into the presence of Cb in livestock, milk and dairy products, Cb surviving possibility during the processing of dairy products, pathogenicity of Cb through the inhalation transmission and oral route (extremely in accordance with the studies from 1940 and 1950), and the way of consumption patterns. Eukaryotic cell is particularly needed to achieve the growth of Cb being discussed as an obligate intra-cellular bacterium.[3] Accordingly, no significant identification and enumeration of the organism have been obtained by means of traditional (reliable) approaches.[4] Cattle, sheep, and goats are recognized as the most important reservoirs of the pathogen.[4] It has been noted that this pathogen is well-founded in urine, feces, milk, and birth fluids of the infected animals. Transmission of infected aerosols probably happens through the human inhalation.[4] Meanwhile, consumption of unpasteurized milk and cheese is less effective in the human infection. *Coxiella burnetii* is considered to be the most important pathogen frequently detected in ticks. Moreover, their role regarding human infection appears to be ill-defined.[5,6]

Since the 2007 epidemic in Netherlands, the global investigation into the field of Q fever attracted considerable interest.[7,8] Notwithstanding the unreliability and lack of data, the provided epidemiological evidence over the industrialized country confirmed that ingestion of raw milk may cause Q fever in some cases. Recent findings regarding above issue were in Michigan, United States, consisting five cases.[4,9]

Kope (pot) cheese is a traditional cheese with the highest production in Greece, Turkey, and Iran (named as a Kope or Coupe cheese in the northwest of country).[10] Traditionally, Kope cheese is the oldest cheese in Iran made up of raw milk.[10] By exploiting raw milk in production progress, the possible cause for Q fever in humans and coxiellosis in animals may thought to be zoonotic pathogens like Cb in Kope cheese.[4,10]

***Correspondence:**

Abdolghaffar Ownagh. DVM, PhD
Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: a.ownagh@urmia.ac.ir

Enhancing the sensitivity of Cb identification has been performed through polymerase chain reaction (PCR) utilizing particular primers targeting transposon-like element sequence (*IS1111*) of the agent.[11-13] The present investigation was intended to study the outbreak of Cb in Kope cheese (for the first time in the northwest of Iran) and cattle milk from dairy farms of Mahabad, Urmia, Miandoab, and Oshnavieh, Iran.

Materials and Methods

Sample collection. The study was carried out on 240 samples of Kope cheese collected from cheese makers in the northwest of Iran and 560 cattle milk samples provided from the south of West Azerbaijan province, Iran, in 2020 (Fig. 1). The animals in the study were grouped based on age into four different groups (2-4, 4-6, 6-8, and over 8 years old). Samples were collected aseptically, placed in a cooler box with ice packs, and immediately transferred to the laboratory.

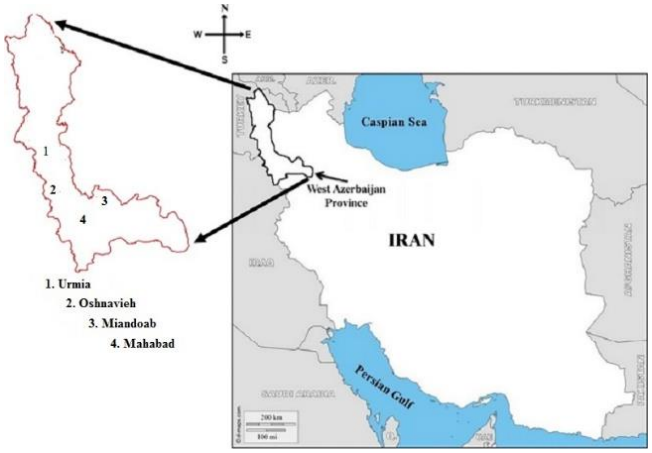

Fig. 1. The schematic map of the study areas in West Azerbaijan province, Iran.

DNA extraction. To extract DNA from milk, 1.00 mL of milk sample was centrifuged for 10 min according to the procedure described by Berri *et al*.[14] The Kope cheese samples were fractionated into smaller portions of 50.00 g, wrapped in sterile bags (Saniplast Mehr, Tehran, Iran) for solid or liquid samples sealed, identified, macerated, and homogenized. The procedure was performed according to the method outlined by De Medici *et al*.[15] During DNA extraction procedure, diethylpyrocarbonate-treated water (DENAzist, Mashhad, Iran) was used as a negative control. The quality and amount of extracted DNA were evaluated by a spectrophotometer (Nano Drop 2000c; ThermoFisher Scientific, Waltham, USA), and extracted DNA samples were kept at – 20.00 ˚C for the later use.

***Coxiella burnetii* touchdown nested PCR.** To perform the steps in the first stage, the method of Parisi *et al*.[13] was used for the first stage of PCR. The PCR reactions (25.00 µL) were prepared as follows: Five microliters of DNA template (10.00 ng of DNA template per reaction), one microliter of each primer with a concentration of 25.00 µM (Trans 1 and Trans 2), MasterMix reactions (12.50 µL), and 5.50 µL of distilled water. In the Trans-PCR stage, PCR touchdown was used to optimize, reduce contaminants, remove inhibitors, and also increase the specificity and sensitivity of the reaction. The temperature program of the touchdown method is shown in Table 1. All oligonucleotides were commercially synthesized by SinaClon (CinnaGen, Tehran, Iran). The primers sequences were previously described by Berri *et al.* and Parisi *et al*.[11,13] The nested-PCR/PCR programs were done according to the methods described by Parisi *et al*.[13] Primers (261F and 463R) were used for the second stage of PCR (nested-PCR). Nested-PCR was performed according to the method described by Khademi *et al*.[16] It should be noted that in each experiment, positive and negative controls were used. All conditions of this step such as mixture of PCR reagents, time and temperature schedule were performed according to the Table 1. Nine Mile strain RSA 493 was used as a positive control of DNA amplification procedure. In this research, bacterial DNA was directly extracted from Kope cheese, since *Coxiella* cannot grow on usual microbiological media and needs specific cell cultures in biosafety level 3 laboratories. The PCR products for both stages were electrophoresed on a 2.00% agarose gel containing safe stain (ENDURO™; Labnet International Inc., Edison, USA) and then, visualized using genius Gel Documentation (Syngene Bio-Imaging, Cambridge, UK), (Fig. 2).

Statistical analysis. The obtained data were statistically analyzed by Chi-square test using Statistical Package for the Social Sciences (SPSS) Software (version 22.0; IBM Corp., Armonk, USA). The *p* value less than 0.05 was considered significant.

Table 1. Primer sequences for detection of *Coxiella burnetii IS1111* gene by nested-polymerase chain reaction (PCR).[13]

Protocol	Primer name	Sequence 5'----3'	Product size (bp)	PCR condition (cycle No.)
Touchdown and Trans-PCR	Trans 1	TATGTATCCACCGTAGCCAGTC	687	95.00 ˚C for 3 min, 94.00 ˚C for 30 sec, 62.00 - 66.00 ˚C (5) for 30 sec, 72.00 ˚C for 1 min, and 72.00 ˚C for 10 min (35)
	Trans 2	CCCAACAACACCTCCTTATTC		
Nested-PCR	261F	GAGCGAACCATTGGTATCG	203	95.00 ˚C for 3 min, 94.00 ˚C for 30 sec, 54.00 ˚C for 20 sec, 72.00 ˚C for 1 min, and 72.00 ˚C for 10 min (35)
	463R	CTTTAACAGCGCTTGAACGT		

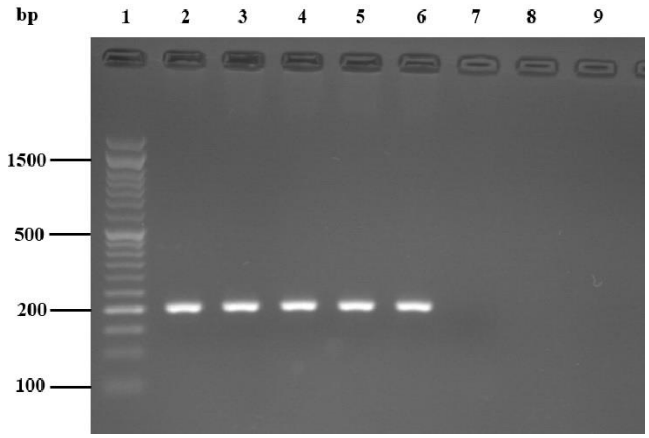

Fig. 2. Agarose gel image of amplified fragment of *Coxiella burnetii* (Cb) *IS1111* gene (203 bp) using nested-PCR. Lane 1: 50-bp molecular ladder (SMOBIO Technology Inc., Hsinchu, Taiwan); Lane 9: Negative control; Lanes 7 and 8: Negative samples for Cb; Lanes 3- 6: Positive samples for Cb; Lane 2: Positive control (Cb standard Nine Mile strain RSA 493).

Results

***Coxiella burnetii* detection.** Out of a total of 800 samples of cattle milk and Kope cheese, 103 samples were positive for the *IS1111* gene.

***Coxiella burnetii* prevalence.** The prevalence of Cb in all cattle milk and Kope cheese samples was 12.50% (95.00% confidence interval [CI]: 11.20 - 16.00%). It was found that the prevalence of Cb in cattle milk and Kope cheese samples were 13.00% (95.00% CI: 10.00 - 16.10%), and 12.50% (95.00% CI: 9.00 - 17.30%), respectively (Table 2).

DNA sequencing. The Cb species-specific DNA segment identification was demonstrated through DNA sequences and GenBank® confirmed the consigned accession numbers (MW541069, MW541070, and MW541071). Despite the fact that the infectious and heat resistant pathogen has been reported in cheese from other countries, the content mentioned above was the preliminary discussion regarding the aforesaid pathogen in a ready-to-eat raw-milk Kope cheese in Iran.

Discussion

The Q fever is among the most commonly discussed diseases in animals being attracted by serological and molecular investigations.[16-18] The important role of ruminants in the mentioned disease is a common knowledge. Therefore, infection originated from consumption of unpasteurized cow milk could be attributed to excreted Cb into the milk. It has not yet established whether consumption of cheese causes the infection or not.

Numerous investigations have revealed Cb DNA in milk and derived products, including cheese, cream, butter, and yoghurt from cows, goats, and sheep.[19] A molecular investigation performed on the most traditional and oldest type of raw-milk cheese in Brazil, known as Minas artisanal cheese being manufactured from bovine milk, has revealed a high prevalence of Cb in this ready-to-eat product, and estimated that 1.62 tons of cheese produced daily is contaminated with this bacterium.[19] *Coxiella burnetii* has been isolated from unpasteurized bovine milk, including the milk being commercialized in the United States. Molecular studies have suggested that the Cb genotypes predominating in dairy products are the same as those infect dairy cattle.[20]

This study focused on Kope cheese produced in the northwest of Iran, and the milk of cattle living in the south of West Azerbaijan province, Iran; the mentioned region has the potential of cattle breeding. The analysis of this study demonstrated that 12.90% of the total milk and cheese samples were contaminated with Cb.

A serological survey of a cohort of goat farmers and workers, and their contacts, involved in an outbreak of Q fever in the Canadian province of Newfoundland, identified the consumption of a cheese made with pasteurized goat milk as a significant independent risk factor for infection. Likewise, a 2-year epidemiological evaluation conducted in 1,200 hospitalized children in Greece found that eating raw cheese coming from rural areas enhanced the risk of Q fever (p = 0.04; odds ratio: 6, and 95.00% CI: 1.10 - 33.20%).[21]

Table 2. Prevalence of *Coxiella burnetii* in cattle milk collected from dairy farms and contamination of Kope cheese samples collected from West Azerbaijan province, Iran.

Parameter	Positive / Total sample	Positive / Total sample	Positive / Total sample	Positive / Total sample
Seasons	**Spring**	**Summer**	**Autumn**	**Winter**
Cattle milk sample	21 / 140 (15.00%)	38 / 140 (27.14%)	12 / 140 (8.57%)	2 / 140 (1.42%)
95.00% confidence interval	10.00 - 21.80%	20.50 - 35.00%	5.00 - 14.40%	0.40 - 5.10%
Region	**Mahabad**	**Urmia**	**Miandoab**	**Oshnavieh**
Cattle milk sample	26 / 140 (18.57%)	14 / 140 (10.00%)	16 / 140 (11.42%)	17 / 140 (12.14%)
95.00% confidence interval	13.00 - 25.80%	6.10 - 16.10%	7.10 - 17.80%	7.70 - 18.60%
Age	**2 - 4 years**	**4 - 6 years**	**6 - 8 years**	**Over 8 years**
Cattle milk sample	7 / 130 (5.38%)	11 / 137 (8.02%)	18 / 143 (12.58%)	37 / 150 (24.66%)
95.00% confidence interval	2.60 - 10.70%	4.50 - 13.80%	8.10 - 19.00%	18.50 - 2.10%
Region	**Mahabad**	**Urmia**	**Miandoab**	**Oshnavieh**
Cheese sample	9 / 60 (15.00%)	4 / 60 (6.66%)	5 / 60 (8.33%)	12 / 60 (20.00%)
95.00% confidence interval	8.00 - 26.10%	2.60 - 15.90%	3.60 - 18.00%	11.80 - 31.80%

A study in the United States showed the occurrences of 10.70% and 0.70% of the infection between different populations. Similar results have also been reported from England, Bulgaria, Slovakia, and Spain.[19,20] According to a study conducted in Sweden in 2007, of 359 cow milk samples collected from cheese factories, 17 samples (7.40%) were contaminated with Cb.[21] In a similar study done in Turkey, 3.50% of the 400 milk samples from 23 herds of sheep were reported to be positive for Cb.[22] In another study conducted in the United States in 2010, of 21 milk samples, nine samples were reported to be positive for Cb.[23] Also, the prevalence of 56.60% of Cb in the cow milk samples was reported using PCR.[17] Studies from various geographical areas of Iran have revealed prevalence of 0.00 - 48.00% in milk samples. For instance, the rates of contamination in the central (Chaharmahal and Bakhtiari province), southern (Jahrom), West Azerbaijan, northwest (Bonab) and southwest (Khuzestan province) areas of Iran were 6.20, 11.00, 16.90, 26.00, and 1.10%, respectively.[24] Moreover, in the study of Khademi *et al*., in 2020, the contamination rate of sheep and goat milk was 7.60 and 16.60%, respectively.[16]

The most important reasons that could be cited for the difference reported in the prevalence of Cb in dairy products in different parts of the world are the diversities in climate and environments of the geographical areas, the type of survey, the type and number of samples, and the season in which sampling took place.[20,25,26]

Taken together, the results of the study highlighted that the age is crucially associated with. Cb in the cattle milk. The current study corroborates the reported results showing the considerable risk factor of age in shedding of Cb into the cattle milk.[18] Current findings revealed that regional diversity is a remarkable factor attributing to the presence of Q fever agent in unpasteurized milk. The results of the present study showed that there was a significant regional variation in the shedding of Q fever agent in raw milk. Therefore, it might be speculated that the population of buffalo, cattle and sheep shedding the bacterium will increase the positive samples.[27]

A satisfactory explanation for the potential of Cb in forming a spore-like environment is resistant small colony variant type of the bacterium. This factor could be responsible for survival of bacteria in milk and dairy products in a long-term period. In contradiction with unpasteurized milk, the revealed results were in line with Cb scarce viability detected in unpasteurized cheese. Moreover, in contrast with unpasteurized cheese, the findings confirmed the vital epidemiological evidence provided by unpasteurized milk for human cases.[3] The conducted study in 2013 has expressed the absence of Cb viability in PCR-positive milk products.[28] Moreover, no risk has been reported for consumers. Detection of Cb viability in hard cheese after eight months of ripening process would be a worst-case scenario.[29] However, only a few studies took a step further toward the investigation of its viability and hazard. Viable Cb was proven in raw cheese by culture in Vero cells and inoculation in mice.[29]

There has been disagreement concerning application of milk heat treatment on Kope cheese production. Therefore, it is recommended that minimum conditions should be undertaken for farmers manufacturing cheese from unpasteurized milk in the following areas: Positive individuals or herds identification through serological or PCR examinations, keeping the herds in closed form, health certification requirements when buying animals, immunization, prenatal separation and removal of placentas and fetal complications.[4]

The Kope cheese is considered as a minimally processed food. Although several authors have called into question the determination of Cb as a foodborne pathogen, this work led us to conclude that Cb risk to humans through the use of raw milk and dairy products could be considered significant.

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Conflict of interest

The authors declare no conflict of interest.

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