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Detection of Coxiella burnetii by Real-Time PCR in Raw Milk and Traditional Cheese Distributed in Tehran Province

Nazanin Mosleh[1], Maryam Moslehishad[2*], Farhad Moosakhani[3]

[1]*Nutrition and Food Sciences Research Center, Medical Sciences Tehran, Islamic Azad University, Tehran, Iran*

[2]*Department of Food Science and Technology, Safadasht Branch, Islamic Azad University, Tehran, Iran (moslehishad@safaiau.ac.ir)*

[3]*Department of microbiology, Islamic Azad University, Karaj Branch, Karaj, Iran*

Abstract

Coxiella burnetii is common causative agent of Q fever in humans and animals. Although the main route of human infection is through inhalation of contaminated aerosols (dust), but oral transmission through contaminated raw milk and dairy products are also possible routes of infection. The aim of this study was to identify and determine the extent of contamination and prevalence of Coxiella burnetii in raw milk and traditional unpacked cheese collected from distribution centers of Tehran province. In this study, 100 samples of raw milk and 40 samples of traditional unpacked cheese were collected from 4 districts of Tehran randomly and then the presence of Coxiella burnetii was assessed using Real–Time PCR method. Our findings indicated that 10 of 100 samples of raw milk were positive for Coxiella burnetii (10 percent) and the rest of them were negative and also 3 of 40 samples of traditional unpacked cheese were positive for Coxiella burnetii (7.5 percent) and the remaining samples were negative. The results of this study showed that bovine milk is one of the potential reservoirs of Coxiella burnetii. Regarding the expand distribution of raw milk all over Tehran, the control of the bacterium in these products for human health should be considered. In traditional unpacked cheeses with high rates of bacterial contamination, the products are another risk (hazard) factor for consumers. According to the results of this study, distribution of raw milk and traditional dairy products is high risk for human health. Therefore, it is important to inform consumers about the potential risks of unprocessed milk and advise them to avoid consumption of traditional milk products.

Keywords: Coxiella burnetii, Raw milk, Real-Time PCR (Polymerase Chain Reaction), Traditional Cheese

Corresponding author: Maryam Moslehishad, Department of Food Science and Technology, Safadasht Branch, Islamic Azad University, Tehran, Iran.
Tel: +982165435516; Fax: +982165433530
Email address: moslehishad@safaiau.ac.ir

Introduction

Q fever, caused by Coxiella burnetii, is a disease with a particular importance due to the prevalence in the animals and consequently in human. It is a major threat to global health, therefore, a survey of diagnosis, identification, transmission routes, and its treatment considered to be a health priority (Angelakis and Raoult, 2010). The Q fever spreads throughout the world and it is caused by Coxiella burnetii, which is a gram-negative obligate intracellular bacterial pathogen, in the Rickettsia family (Arora, 2001).

This disease is a febrile illness with symptoms similar to the flu and has acute and chronic stages. Symptoms of this disease in the acute phase include fever, chills, severe headaches, muscle pain, and the appearance of cutaneous grains and in the chronic phase include; endocarditis (inflammation of the inner layer of the heart muscle), liver disorders hepatic disorders and It also causes hepatitis (Arricau-Bouvery and Rodolakis, 2005).

Bovine and sheep are the main hosts of this disease, but many other animal species can become infected and excrete the organism via milk. Coxiella burnetii is transmted to humans by inhalation of dust, direct contact with livestock, drinking raw milk or other non-pasteurized milk products (Bashiribod et al., 1976).

Recently, the Q fever, as an emerging disease, has spread to some countries. For example, in the east of Holland, the center of goat breeding, three major epidemics have occurred. To control the disease, they eliminate the infected goat and sheep since 2007. Extensive vaccination has been done from 2009 to breeding season in 2010 (Berri et al., 2007).

Coxiella burnetti enter the body of domestic animals, firstly via direct contact, secondly through the bloodstream. It enters into various tissues of the body, particularly in breast tissue, extra-mammary lymph nodes, pairs, reproductive and intestinal system. Finally, it gets transmitted through contaminated milk to humans (Bosnjak et al., 2010).

The objective of the present study was to determine the prevalence of Coxiella burnetti in unpasteurized milk and cheese samples using Real-Time PCR assay in Tehran. To our research no such study has been performed until now.

Materials and Methods

Sampling:

In the present research, 100 raw milk samples and 40 unpacked cheese samples were obtained from Tehran. For this purpose, the city of Tehran was divided into 4 districts: northwest, northeast, southeast, southwest, then samples were randomly collected in sterile transport containers from each area. The amount of sample was one kilogram of raw milk and half a kilogram of unpacked cheese from the supplier's dairy stores. Samples were taken from each area equally (from each region, 25 raw milk samples and 10 non-pasteurized and unpackaged cheese)

DNA Extraction:

Milk samples were homogenized in sterile containers and then 1.5 mL of each milk sample was transferred into the microtube. Then, centrifuges were carried out at 14000 rpm for 10 min. TaqMan Universal kit from Life Technology Company was used to extract, based on the kits method. The milk samples were converted into three phases including a fat layer, blue phase and sediment after centrifuge. Then the fat layer and the blue phase were removed from the sediment. On the remaining residue, 180 μL of lysis buffer and 20 μL proteinase K were added and completely homogenized on the shaker and placed in Bain Marie at 55 ˚C for 30 min.

For cheese samples, 0.9 g of each sample was weighed in a microtube. 180 mL lysis buffer and 40 μL of proteinase K were added to each sample and they were completely homogenized. Then it was placed in Bain-Marie at 65 ˚C for 3 h. Then, It was centrifuged for 10 min at the speed of 12000 rpm. The top phase was taken to continue the experiment at a later stage. The rest of the experiment was the same for milk and cheese samples. 360 μL binding buffer was added to the samples from the previous stage and homogenized again. Then the samples were transferred to the Bain-marie at 70˚C for 10 min. Then the samples were extracted from Ben-Marie and added to them 270 μL of 96% ethanol and completely homogenized. The

samples were transferred to a filtered microtube and centrifuged for 1 min at 1000 rpm. The solution under the filter was discarded. The DNA contained in the samples is trapped on the filter and 450 μL of wash buffer solution was added and centrifuged for 1 min at 1000 rpm. And the sub-filter solution was discarded. The washing step was repeated twice. Then the drying step was performed. For this purpose filtered microtube was centrifuged for 2 min at a speed of 14000 rpm. The filtered microtube was placed on another microtube. 50 μL of elution buffer was added to that. It was placed at room temperature for 3 min. Then it was centrifuged for 1 min at 1000 rpm. The extracted DNA was collected from the bottom of microtubes .

Real -Time PCR:
The method was performed as follows: The samples and three controls (positive control, negative control, and NTC control) were placed in a special metal rack. (Table 1).

Table 1: Quantity of components of TaqMan Universal kit

TaqMan Universal kit	Quantity
Master mix	μl 2.5
Primer CoxbS	μl 2
Primer AsbS	μl 2
Probe CoxP	μl 0.75
Distilled water	2.75 μl

20   μL of TaqMan Universal master mix kit (Table 1) was divided into equal aliquots to microplates. No-template control (NTC) as an indicator of contamination contains all essential mixture components except sample. To prepare sample test, 5 μL DNA samples extracted based on Molecular Biological System Transfer (MBST) kit method was added to each of microtube containing the master mix. Nega-tive control was prepared by addition of 5 μl of distilled water to the mixture. Positive control was prepared by addition of 5μl from positive sample to the mixture components. Fianally, the lid of microtubes closed and placed inside the rotating plate of the device.
The Coxiella burnetti thermal program is given to the software program of the device as shown in Tables 2 and 3.

Table 2: Thermal program for Real-Time PCR

95ºC		15 min (heat activation of Taq)
Then 40 cycles		
	a.	94ºC 15 sec
	b.	60ºC 60 sec

Table 3: Primer and probe used in Real-Time PCR

primer CoxbS	GATAGCCCGATAAGCATCAAC
primer AsbS	GCATTCGTATATCCGGCATC
probe CoxP	FAM-TCATCAAGGCACCAATGGTGGCCA-BHQ-1

Finally, samples with a Ct of 15 to 35 were positive, and samples with a Ct of less than 15 were reported negative.

Statistical Analysis

For analysis of samples, SPSS 22.0 software (SPSS Inc, IBM, Chicago, IL) was used. Chi square test was applied to compare the significance level between different geographical areas, for milk and cheese; two by two comparisons were done between different regions.

Results and Discussion

Coxiella burnetii is the causative agent of acute Q-fever in human and the disease can be transferred through non-pasteurized milk and milk products such as cheese. Although hygienic principles and pasteurization of milk and milk products are performed in Iran for many years, consuming unpasteurized fresh milk and traditional dairy products is also common especially in recent years. Due to the convenience, high speed, precision and high sensitivity of the Real-Time PCR method to detect Coxiella burnetti, this method was used to diagnose infection in raw milk and milk products in the centers supplied in Tehran.

This method had been used successfully in research performed by Khalili et al. (2015) in Iran as well as research conducted in France, Belgium, Australia, and Italy, which can confirm the correctness of the research method (Banazis et al., 2010, Capuano et al., 2012). This study was performed to investigate the contamination of Coxiella burnetii in the traditional centers of milk supply in Tehran in 2016. After obtaining raw milk and non-pasteurized cheese samples in Tehran and analyzing the samples by Real-Time PCR, the results showed that 10% of raw milk samples and 7.5% of cheese samples were positive, so the contamination was confirmed by the presence of Coxiella burnetii in livestock farms in Tehran. The number of positive samples and the percentage of prevalence are different among different regions, which the most positive raw milk samples were obsereved in the northwest region of Tehran and the southeastern region has the lowest positive samples (Table 4). The northeast region had the highest positive cheese samples and the northwest region had the lowest positive cheese samples. Therefore, the prevalence of Coxiella burnetti in the northwest of Tehran was more than total prevalence rate, and the northeast region had a slightly different percentage of total prevalence (Table 5).

Table 4: Comparison of raw milk contamination with Coxiella burnetti by region

Region	Positive percentage	negative percentage
North West	ND[*]	100
North East	14.3	85.7
South East	11.1	88.9
South West	11.1	88.9

•*Not Detected*

Table 5: Comparison of non-pasteurized and unpacked contamination cheeses with Coxiella burnetti by region

Region	Positive percentage	Negative percentage
North West	23.7	76.93
North East	12.5	87.5
South East	ND[*]	100
South West	3.7	96.3

•*Not Detected*

In the regions where the prevalence of the Coxiella burnetti was the most, this was likely related to livestock farms that supply these areas and maybe the prevalence rate of Coxiella burnetti was high in this livestock.

Although Q fever is primarily an occupational disease, consumption of contaminated milk and dairy products can also play an important role in the epidemiology of disease in human beings. (Tajbakhsh, 2005).

According to the studies in the United States, France, Belgium, Australia and Italy, the presence of contamination by Coxiella burnetti was confirmed in these countries (Banazis et al., 2010, Capuano et al., 2012).

In Iran, regarding the presence of bacterial contamination with Coxiella burnetti in some parts of the country such as Fars, Yazd, Khorasan Razavi and Kerman, as well as neighboring countries, and considering the strength of the organism and the ways of easy transmission of the organism, the probability of infection in the central region of the country seems to be strong and this study confirmed the contamination (Khanzadi et al., 2014, Khalili et al., 2015).

Khademi et al (2015) studied the prevalence of Coxiella burnetti in sheep's raw milk by a Nased-PCR method in Chagni and Pol-dokhtar from Lorestan province in 2015. The positive samples were 24 of 68 sheep's raw milk (35.29%). This study showed that sheep's raw milk could be one of the potential reservoirs of Coxiella burnetti in Iran (Khademi et al., 2015). In this research, the obtained data revealed that the percentage of prevalence of Coxiella burnetti was different in regions due to the geographical area and the rate of contamination of livestock farms and the difference with the present research can be the type of host in addition to the mentioned cases. The prevalence of Coxiella burnetti in cattle farms in Ajabshir by Trans-PCR method was studied by Khademi et al (2014). The results showed that 25 percent of raw milk samples were positive and bovine milk can be a potential reservoir of detriment in Iran (Khademi et al., 2014).

According to the study by Kargar et al. 100 samples of cow milk were assessed in 2012 using Real-Time PCR method in the Jahrom city in Fars province of Iran, the prevalence of Coxiella burnetti was 11 percent (Kargar et al., 2012).

Khalili et al., in 1394 in southeastern Iran, conducted a research on clinically healthy goat herds. They sampled 31 milk specimens from goat farms in Kerman and tested for the presence of Coxiella burnetti by the Trans-PCR method. In this study, 5 samples of 31 samples (16.12%) were positive; therefore, the results showed that healthy milk was one of the potential reservoirs of Coxiella burnetti in this region (Khalili et al., 2015).

Khan Zadi et al. (2014) in southeastern Iran in 2014 conducted a research on goat herds. They analyzed 147 commercial and traditional dairy products including 28 kinds of cheese, 26 yogurt samples, 23 sheep's milk samples, 60 cows milk samples and 10 goat milk samples by Touch-Down PCR method from the supply centers of Mashhad-Khorasan Razavi province. In this study, 2 samples of 28 cheese samples (7.14%), 2 samples of 26 samples of yogurt (7.69%), 8 samples of 23 samples of sheep milk (34.7%), 2 samples of 60 milk samples of cow (3.33%) were positive. 10 goat milk samples were negative in this identification. Consequently, the results showed that non-pasteurized raw milk of cows, goats, and sheep were the main reservoir of Coxiella burnetti.

A study was performed by Kim et al. (2005) in the United States, 316 herds were randomly chosen and sampled from their reservoir and tested by PCR to evaluate the presence of Coxiella burnetti. Finally, the results revealed that 3.94% of the samples contained Coxiella burnetti (Kim et al., 2005). In this study, the raw milk samples of the bovine reservoir were positive.

Anastácio et al. in 2016, in Portugal, reported the prevalence of Coxiella burnetti 30.9% in bulk milk tank sample and ruminants were identified as the main reservoirs of Coxiella burnetti (Anastácio et al., 2014). Banazis et al. in Australia in 2010, the prevalence of Q fever in 329 cows samples and 50 sheep samples were assessed by ELISA. It showed that the prevalence of cows were 0.61% and sheep were zero percent (Banazis et al., 2010).

Gulmez Saglam- and Sahin, in 2016 using the Trans-PCR method in Turkey's Kars, studied 350 samples of bovine milk and 250 sheep milk samples. The prevalence of Coxiella burnetti was reported as 42.1% and 0.04%, respectively. The results of this study showed the presence of

Q fever in cow and sheep in the Kars area (Gulmez Saglam and Sahin, 2016), even though the contamination level of sheep was very low in this area.

In the similar studies in Iran and the other countries, differences in the percentage of prevalence might be due to the geographic region, more accurate test method, level of contamination in livestock farms and the host type.

Conclusion

The obtained data revealed a significant prevalence of Coxiella burnetti as the causative agent of Q fever in raw milk and unpacked and non-pasteurized cheeses in dairy supply centers in Tehran. Therefore, it can be a risk for public health in Tehran and this is one of the most important reasons not to consume raw milk and dairy products from traditional suppliers. Since most people do not have enough knowledge about this matter, the infection might be transmitted using these products and this may be the cause of disease among human populations. In Tehran, there was a significant difference in the prevalence of Coxiella burnetti related to livestock farming, which supplies the raw milk. Consequently, animal health monitoring systems should be implemented in dairy cattle livestock as well as the different reseons for prevalence of Coxiella burnetti should be determined.

Based on the results of this study and other research, it is observed that the prevalence of Coxiella burnetti is not the same in each region due to the route of transmission of this disease, the geographical area and the rate of contamination of livestock farms. Additionally, the type of host, sample number, and test method are also effective in the results.

Acknowledgments

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