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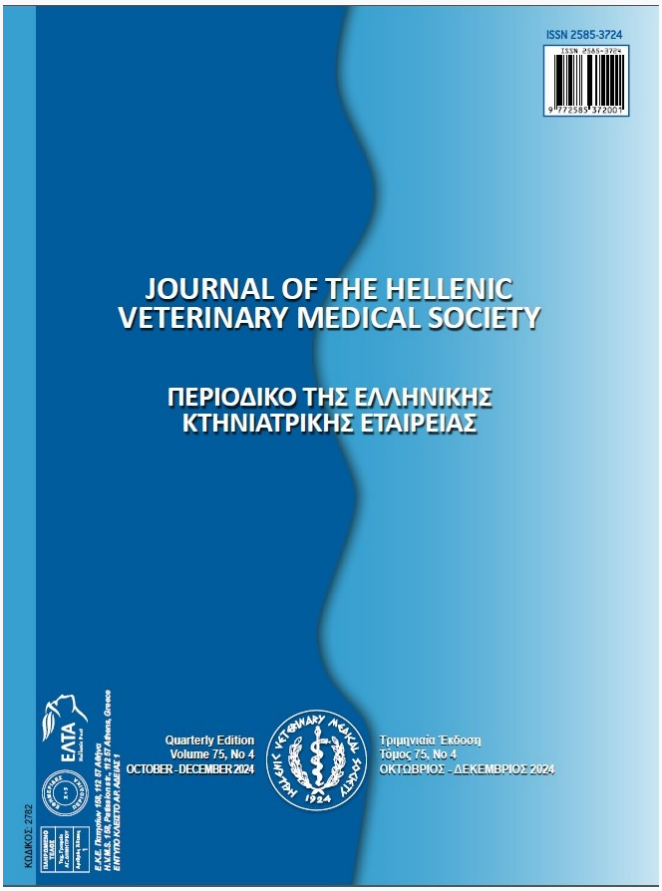

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Research article
Ερευνητικό άρθρο

Epidemiology of Q fever in milk by using nested-PCR targeting the repetitive elements *IS1111* and *COM1* genes in *Coxiella burnetii* in Iran

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ABSTRACT: Q fever, which is caused by *Coxiella burnetii*, distributes worldwide. The main reservoirs of the disease are small ruminants. However, many species of animals can be infected. Humans and animals get Q fever when they breathe in contaminated aerosols accompanied by *C. burnetii*. The excreted bacterium in the milk could infect animals and humans. In this cross-sectional study, 416 cattle milk samples were collected randomly from dairy herds in Khorramabad, Iran in 2019. The PCR products were examined by electrophoresis using an agarose gel. All milk samples were subjected to DNA extraction and examined by a high specific nested-PCR method targeting two different genes (*IS1111* and *Com1)*. The prevalence of *C. burnetii* in the evaluated samples using *Com1* and *IS1111* target genes was 5.8% and 14.4% respectively. Quantitative study showed that *IS1111* gene is more sensitive than *Com1* gene and results were 0.6 fg/ml and 0.06ng/ml DNA respectively. The results of this study show that Transposal *IS1111* gene was highly sensitive and useful for the direct detection of *C. burnetii* in milk samples. This technique is a one-step and fast process in comparison to other assays. The overall results of this study indicated that cattle milk can be a potential reservoir for *C. burnetii* in Iran.

***Keywords*:** Cow milk; Nested-PCR; *Coxiella burnetii; Com1* gene; *IS1111* gene

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INTRODUCTION

First cases of Q fever disease were identified among slaughterhouse workers in Australia in 1933 (Ruiz and Wolfe, 2014), with symptoms of fever, headache, and malaise. *Coxiella burnetii* is a Gram-negative, rod-shaped bacterium. *C. burnetti* is the causative agent of Q fever disease in humans and animals (Maurin and Raoult, 1999; Parola and Raoult, 2001). The bacterium *C. burnetii* multiplies mainly in macrophages, the phagolysosomal vacuoles of host cells. The placenta is the ~~goal~~ target organ for *Coxiella* (Eldin et al., 2017; Maurin & Raoult, 1999). Therefore, it is suggested that farm animals and pets are the main reservoirs of infection. The clinical spectrum of the disease varies (Cerf and Condron, 2006; Kim et al., 2005).

Subclinical infection is a main form of the disease in animals with clinical findings like late abortions, stillbirth, delivery of weak neonates, and infertility. Infection in humans is asymptomatic but in acute disease, it can be lead to death (Agerholm, 2013; Maurin and Raoult, 1999). It is suggested that secretion of *C. burnetii* varies in different animals relevant to the excretion path and species, as follows: in ewes and goats 8 and 20 days after lambing, respectively; maximally 8 and 13 days in milk of ewes and cattle, respectively (Agerholm 2013); also goats may shed *C. burnetii* in two consecutive kidding periods (Alvarez-Alonso et al., 2020). In the regions with Q fever problems, raw milk is highly infectious. 10% of people in the United States, who consume raw milk, tested positive for serology however, this rate was 7% among ordinary people (Eldin et al., 2017). Some authors have accepted the argument that the forms of the disease would be different according to the route of contamination: hepatitis for ingestion, pneumonia for inhalation (Plummer et al., 2018). Evidence for infection with Q fever caused by consumption of raw (unpasteurized) milk have been reported in Michigan, America (Cerf and Condron, 2006; Fishbein and Raoult, 1992; Serbezov et al., 1999; Signs et al., 2012).

Milk is an ideal culture medium for bacteria such as Brucella, Listeria, Mycobacterium, and *C. burnetii*, so inappropriate processing of dairy products could be a major problem especially for developing countries. It is of utmost importance to recognize milk-borne bacteria which are transmitted due to improper thermal processing of milk or dairy products (Dhanashekar et al., 2012). Q fever is endemic in animals in Iran but, due to a lack of diagnostic facilities and low level of awareness in the Iranian health care system, it has not been diagnosed for more than forty years (Yaghmaie et al., 2015), The nested PCR approach is being applied for highly sensitive and specific direct detection of *C. burnetii* using *com1* and transposon genes (Lockhart, 2010). There are different genes for identifying and diagnosing *C. burnetii*. The *com1* gene (27 kDa), which codes the outer membrane protein of *C. burnetii*. Other genes include heat shock operons that encode two proteins (*htpA* and *htpB*), isocitrate dehydrogenase (*icd*), and transposon (*Trans*) which represent a repetitive region located in the *C. burnetii* genome (Boldis et al., 2013). Insertion sequence (*IS1111*) in the *C. burnetii* genome is used as a specific target in the diagnosis of *C. burnetii* by PCR, due to the high number of (7-110) copies (Klee et al., 2006).

More recent investigations have provided considerable insight into the prevalence of *C. burnetii* in the milk of cattle, buffaloes, sheep, and also goats in Iran (Esmaeili et al., 2019; Khademi et al., 2015; Khademi et al., 2019; Khademi et al., 2020). Previous studies, showed a different prevalence of *C. burnetii* with *IS1111* and *Com1* genes obtained from cattle milk (Berri et al.,2000; Berri et al.,2003; Parisi et al., 2006; Esmaeili et al., 2019). Therefore, the present study aims to compare the sensitivity of *Com1* and *IS1111* genes in the detection of *C. burnetii* infection in milk.

MATERIALS AND METHODS

Study area

Lorestan province has the first rank in terms of livestock density in Iran so that the average livestock density per unit area in this province is 220 livestock units per square kilometer and in the country is equivalent to 84 livestock units per square kilometer. Lorestan Province is located in the west of Iran, amidst the Zagros Mountains. This province is one of the most important provinces in the field of breeding domestic ruminants (cattle, sheep, and goats) and the economy of the people living in this region depends on agriculture and animal husbandry. Therefore, many people in this province have a close relationship with livestock (http://safireaflak.ir/fa/news/233) (Fig. 1).

Milk sampling

In a randomized collection approach, 416 samples were obtained from 21 dairy farms in the Khorramabad region during four seasons in 2019. Sampling was performed on apparently healthy cattle. To fur-

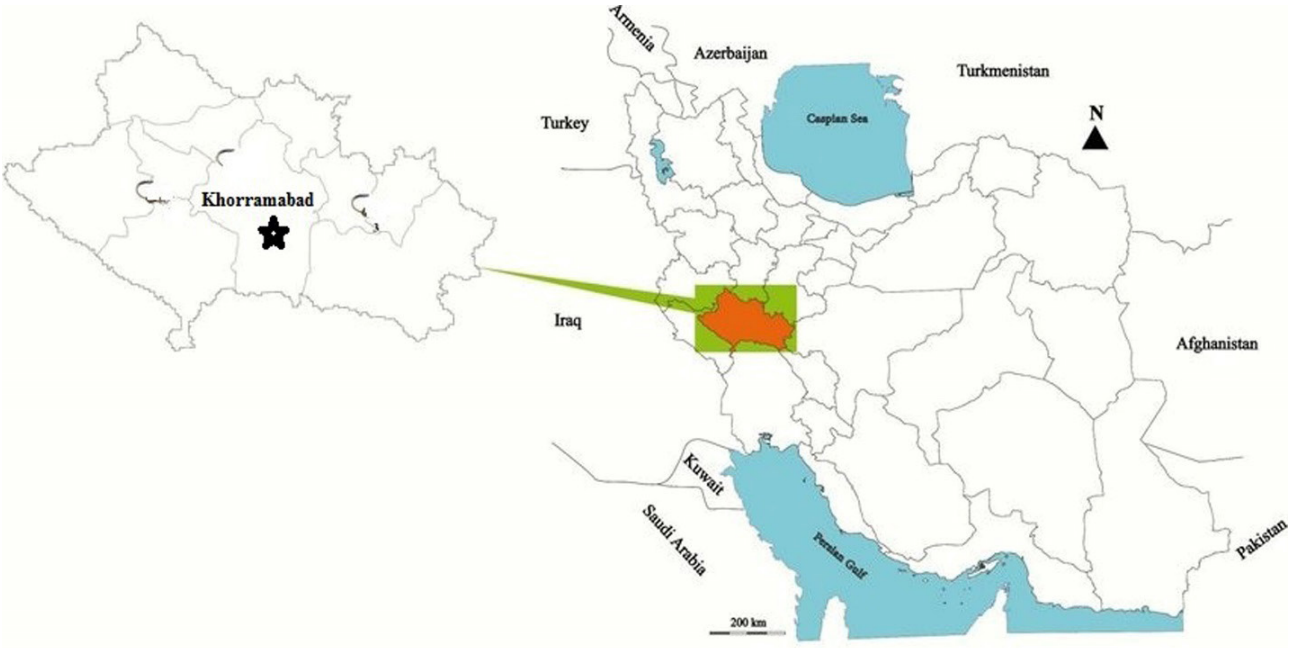

Figure 1: The schematic map of the study areas, Khorramabad region, Iran.

ther processing our research, transportation of obtained milk samples to the microbiology laboratory in Veterinary Medicine Faculty were performed using ice packs.

DNA extraction and nested- PCR assay

To extract DNA from milk, 10 ml of milk sample was centrifuged for 10 minutes (Berri et al.,2000; Berri et al.,2003; Parisi et al., 2006). The pellet was then treated by genomic DNA purification kit (Gene all cell SV mini 250p/Bioneer CO., Korea) according to the manufacturer's instructions. DNA samples were stored at −20°C for further use. The primers utilized in this study were supplied by Cinna GenCo (Iran). The sequence of the primers was previously described by Parisi et al., (2006) and Berri et al., (2000). The primers for *IS111*genes (Trans1, 2 and 261F, 463R) and nested-PCR thermal program were used by methods described previously by Parisi et al., (2006) (Table 1). The primers used for *Com1* genes (OMP1, OMP2, and OMP3, OMP4) were described previously by Fretz et al.,(2007) and Zhang et al., (1998) (Table 1). For nested- PCR, the reaction was performed in a total volume of 25µl containing 5µl DNA sample, 1.5 µl MgCl2, 1 µl dNTPs, 1 µl primer OMP1, 1µl primer OMP2, and 1 µl of Taq DNA polymerase. The second amplification utilized the dilution process conducted in the first amplification. Following this, the temperature and reaction volume was comparable to that used by first amplification.

In this study, DNA was also extracted from *C. burnetii* reference strain Nine Mile II, RSA 493bp, as positive control and Sterile distilled water was used as negative control. PCR product for the *Com1* and *IS1111* gene in the first stage of PCR was 501 and 687 bp respectively. And also in the second stage, PCR products were 438 and 203bp, respectively. For this proposal, twenty samples were measured at a wavelength of 260 for determining DNA concentration, and follow on 280/260 (Protein/DNA) ratio was measured. To better compare and determine the sensitivity of primers, PCR was performed on successive dilutions of DNA extracted from *C. burnetii* reference strain Nine Mile II, RSA 493.

Detection of PCR products

The PCR-amplified products containing *Com1* and *IS1111* genes were examined by electrophoresis (1.5% and 2% agarose gel, respectively), and visualized under UV by gel documentation (Figs. 2, 3). Different primers and specificity of PCR method were used to achieve high sensitivity for the detection of *C. burnetii* infection in clinical samples (milk), then a positive control sample of eight consecutive dilutions was prepared and its values measured with Thermo Fisher Scientific, NanoDrop 2000c Spectrophotometer, USA.

Table 1: Primer sequences for detection of *C. burnetii IS1111* gene and *Com1* gene by nested- PCR

Gene and Primer	Sequence	PCR conditions ºC/ of S				PCR cycles	Reference
		pre Denaturation	Denaturation	Annealing	Extension		
Trans 1	5'-TATGTATCCACCGTAGCCAGTC-3'	95/3m	94/30s	62-66/30s	72/10m	35	Parisi et al., 2006
Trans 2	5'-CCCAACAACACCTCCTTATTC-3'						
261F	5'-GAGCGAACCATTGGTATCG-3'	95/3m	94/30s	54/20	72/10m	35	
463R	5'-CTTTAACAGCGCTTGAACGT-3'						
OMP1	5'-AGTAGAAGCATC CCAAGCATTG-3'	94/3m	94/45s	56/45s	72/5m	30	Zhang et al., 1998
OMP2	5'-TGCCTGCTAGCT GTAACGATTG-3'						
OMP3	5'-GAAGCGCAACAA GAAGAACAC-3'	94/3m	94/45s	56/45s	72/5m	30	
OMP4	5'-TTGGAAGTTATC ACGCAGTTG-3'						

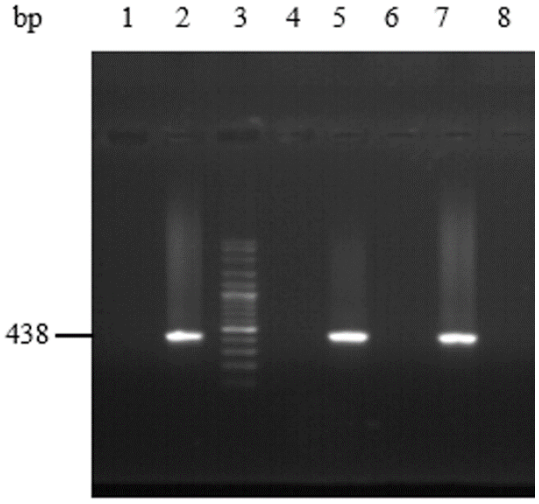

Figure 2. Detection of *C. burnetii* DNA by nested PCR with primers OMP1-OMP2 and OMP3-OMP4. An agarose gel electrophoretogram of amplified DNA after the nested PCR and ethidium bromide staining is shown. Lane 3: molecular size markers 100-bp DNA ladder (Fermentas); the Milk samples in lanes 5, 7, samples positive. (Lanes 4, 6, 8, the samples were negative); lane 2, positive control (*C. burnetii* standard Nine Mile strain RSA 493), lane 1; negative control.

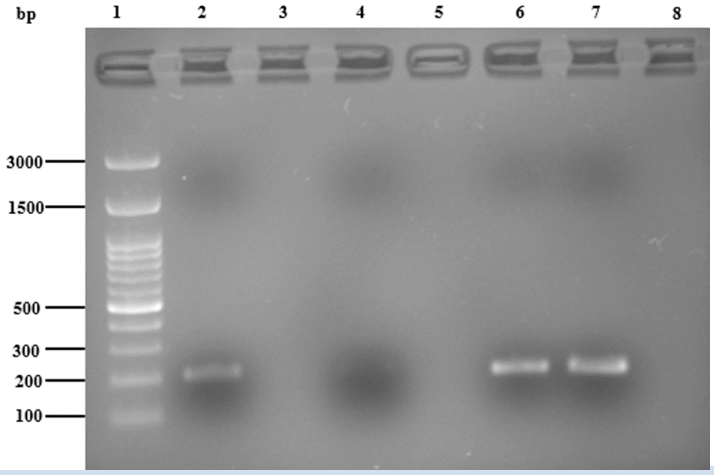

Figure 3: Agarose gel image of amplified fragment of *C. burnetii IS1111* gene (203bp) using nested-PCR. Lane 1, 100-bp molecular ladder (Smobio Technology Inc., Taiwan), Lane 2, Positive control (*C. burnetii* standard Nine Mile strain RSA 493); lanes 6, 7 positive samples for *C. burnetii*, Lane 3, 4, 5, negative samples for *C. burnetii*, lane 8, negative control.

Statistical analysis

The Chi-square approach was adopted to statistically analyze the collected data through the use of SPSS software Ver. 19 (SPSS Inc., Chicago, IL). On average, we found a value< 0.05 for P significant.

RESULTS

In total, 24 (5.8%) of 208 tested samples were positive for *Com1* gene. while, the overall response to 60 (14.4%) of the mentioned samples were positive for *IS1111 gene.* According to this result, there were significant differences using targeted genes in detection of bacterium in milk samples. And also, results showed significant differences in *C. burnetii* prevalence seasonally. Detailed information on *C. burnetii* prevalence in cattle milk samples in the Khorramabad region were showed in (Table 2).

In this study, Nested- PCR which was used to detection of *IS1111 gene* were very high sensitive and the lowest detection rate was about 0.6 femtograms of DNA per milliliter. While, the lowest detected DNA concentration by nested- PCR to the *Com1* gene was 0.06 ng/ml (Table 3).

Table 2: Prevalence of *C. burnetii* in cattle milk based on *Com1* and *IS1111* genes.

Region	Season	The number of samples per season	*Com1* gene Number (%) C. *Burnetii* positive sample (95% CI :)	*IS1111* gene Number (%) of *C. Burnetii* positive sample (95% CI :)
Khorramabad	spring	104	6(5.8%) (2.7%-12%)	12(11.5%) (6.7%-19%)
	summer	104	12(11.5%) (6.7%-19%)	34(32.7%) (24.4%-42.2%)
	autumn	104	4(3.9%) (1.5%-9.5%)	8(7.7%) (4%-14.5%)
	winter	104	2(1.9%) (0.5 %-6.7%)	6(5.8%) (2.7%-12%)
Total		416	24 (5.8%) (95% CI: 3.9%-8.6%)	60 (14.4%) (95% CI: 11.4%-18.1%)

Table 3: Minimum DNA concentration detection of *C. burnetii* based on *Com1* PCR, Trans-PCR and Nested-PCR.

primer		The lowest DNA concentration detection							
		6 ng/ml	0.6 ng/ml	0.06 ng/ml	6 pg/ml	0.6 pg/ml	0.06 pg/ml	6 fg/ml	0.6 fg/ml
Com1	PCR	+	+	+	-	-	-	-	-
	nested-PCR	+	+	+	+				
IS1111	PCR	+	+	+	+	+	-	-	-
	nested -PCR	+	+	+	+	+	+	+	-

DISCUSSION

In the past decade, researchers in several countries have sought to indicate the importance of Q fever outbreaks on global public health threat. Q fever outbreaks have received much attention over the last decades in several countries (Eldin et al., 2017). Accordingly, further investigation would be needed to determine exactly how Q fever outbreaks affect global public health. The variation of *C. burnetii* prevalence in raw milk samples has been reported from 0% up to 95%, following the studies performed in various countries (Pexara et al., 2018). The aim of this study was to evaluate the sensitivity of PCR with two different primers for the detection of *C. burnetii* in cattle milk samples. The *IS1111*-PCR showed more positive samples (14.4%), while the frequency of *C. burnetii* using OMP-PCR in determined samples was 5.8%., Furthermore, results showed that all positive samples to *Com1* gene (24 samples) were detected by *IS1111* genes primers. This result demonstrates that PCR with primers Trans1 and Trans2 are found to be highly sensitive and reliable for the detection of *C. burnetii*. Prior studies have noted a widespread distribution of Q fever among cattle in the west of Iran (Esmaeili et al., 2019; Heydari et al., 2021; Khademi, 2015; Khademi et al., 2019; Khademi et al., 2020). Bacteria are excreted into the environment by animal secretions, especially during calving, placenta and fetal fluids, vaginal secretions, feces, urine, and milk (Esmaeili et al., 2019; Khademi et al., 2020). It seems possible that the high prevalence of Q fever in females might be explained by the high susceptibility of females (Berri et al., 2001). In an attempt to limit the spread of the infection, the exposure of humans and animals to shedder ruminants needs to be decreased (Knobel et al., 2013). In the present study the highest preva-

lence of *C. burnetii* shedding in milk was observed in warm seasons which ~~were~~ is in accordance with the previous reports that demonstrated that the increased incidence of Q fever in animals is linked to lambing season. Similarly, in many European countries, the highest numbers of cases were reported during summer due to spring lambing season (Parisi et al., 2006; Pexara et al., 2018).

According to many studies on *Com1* and *IS1111* gene from Iran and other countries, *C. burnetii* is presented in cow's raw milk (Esmaeili et al., 2019; Khademi et al., 2019). There have been many studies performed in other countries indicating the rate of *C. burnetii* prevalence in dairy products such as milk which is almost 94% in bovine milk bulk tank samples in the USA, 4.7% of bovine milk samples in Switzerland, and 3.5% of bovine milk samples in Turkey (Fretz et al., 2007; Kim et al., 2005; Öngör et al., 2004).

In contradiction with conventional PCR, it is previously demonstrated that the nested PCR assay for *C. burnetii* concerning new internal primers for trans-PCR (*IS1111*), can achieve better results (Mares-Guia et al., 2018). According to Klee et al., (2006), the insertion element numbers in the several isolates are presumably to be very variable. The differences in the content of *IS1111* genes are significantly employed in genotyping and phylogenetic analyses of *C. burnetii* isolates (Eldin et al., 2017).

As the focus of the study is on *C. burnetii* DNA detection based on PCR in cattle milk samples, the findings of this study demonstrated that the nested-PCR for *C.burnetti* molecular detection had high sensitivity. Then it is suggested that this method can be enhanced with serological and culture method.

Finally, regarding the public health issue of Q fever, we suggest that serological and molecular surveys on other species of livestock should be carried out in the Khorramabad region and other provinces of Iran to have a clear picture of *C. burnetti* infection in Iran.

CONCLUSION

In conclusion, the results of this study showed that the application of the *IS1111* gene is better than the *Com1* gene in the detection of *C. burnetii* in milk samples and the difference is significant. To obtain reliable results, the authors suggest that large numbers of samples should be analyzed in subsequent studies. Also, it is better to compare different methods for example touchdown-PCR, Real-time PCR, and semi-nested PCR for the detection of *C. burnetii*. The nested-PCR method can be used to diagnose *C. burnetii* in milk and other clinical specimens because of its ease to use and reliability.

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CONFLICT OF INTEREST

The authors have not conflicts of interest to disclose.

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