

## Detection of *Coxiella burnetii* in cows', goats', and ewes' bulk milk samples using polymerase chain reaction (PCR)

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### Abstract

Q fever is a widespread zoonotic disease that is caused by obligate intracellular bacteria, *Coxiella burnetii*. This study was planned to determine the prevalence of *C. burnetii* in cows', goats', and ewes' bulk milk (BM) samples using PCR and to confirm positive results by DNA sequencing. A total of 150 BM samples (50 samples of each cows', goats', and ewes' milk) collected from 15 randomly selected dairy farms in Hatay province were analyzed. The BM samples were taken between January 2012 and July 2013. Bacterial DNA was extracted directly from milk samples. Nine of the BM samples (6 %) were PCR positive; five from cows' BM, two from ewes' BM, and two from goats' BM. Bacterial DNA was detected in 3 of 15 (20 %) dairy farms. Positive results were confirmed by DNA sequencing. The sequencing results of *Coxiella* DNA extracted from cows', goats', and ewes' milk samples were consistent with the reference strain with 100 %, 99.7 %, 99.8 % homology, respectively. BM samples were found to be contaminated with *C. burnetii*; therefore, taking hygienic measures is necessary for food safety and public health.

*Key words:* bulk milk, *Coxiella*, polymerase chain reaction, sequence analysis

### Introduction

Q fever is a worldwide distributed zoonotic disease caused by *Coxiella burnetii* (Hoover et al., 1992; Maurin and Roul, 1999; Norlander, 2000), except New Zealand (Hilbink et al., 1993).

Seroepidemiologic studies have reported different results for the seroprevalence of the bacterium in sheep (3 to 79 %) in Turkey (Özyer et al., 1990; Çetinkaya et al., 2000; Kalender, 2001; Kılıc et al., 2005; Dogru et al., 2010; Kennerman et al., 2010; Parın and Kaya, 2012). These wide differences could be due to sampling region in the country, testing methods, and the year of surveys. The seropositivity of *C. burnetii* in cattle serum samples was 19 % reported by Özyer et al. (1990), 5.8 % by

Çetinkaya et al. (2000), 9.5 % by Seyitoğlu et al. (2006), 12.4 % by Gazyagcı et al. (2011). These data suggest that prevalence of *C. burnetii* infection is higher in small ruminants than in cows in Turkey.

*C. burnetii* infections have been reported in humans, farm animals, pets, wild animals, and arthropods. Cows, goats and ewes are the main sources of *C. burnetii* in human infections. In 2012, according to the scientific report of European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), a total of 643 confirmed human cases of Q fever were reported in the European Union (EU). Almost all reporting Member States found *C. burnetii* in cattle, sheep and goats, which indicates that this bacteria is widely distributed in the EU (EFSA and ECDC, 2014).

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Infected animals shed bacteria into the environment through their urine, feces, milk, and birth products. Humans are infected by inhalation of contaminated aerosols or by consumption of raw milk or dairy products (Kim et al., 2005; Guatteo et al., 2006; Rodolakis, 2009).

Isolation of *C. burnetii* by conventional culture methods has been difficult and time consuming. However, serological tests such as complement fixation, indirect immunofluorescent assay (IFA), and enzyme-linked immunosorbent assay (ELISA) have been routinely used for the diagnosis of disease, but immunological cross-reactions can adversely affect the sensitivity of them. In the present study, rapid and sensitive polymerase chain reaction assay was used with primers based on a transposon-like repetitive region for direct detection of *C. burnetii* in bulk milk samples.

In Turkey, majority of researches have focused on the seroprevalence of *C. burnetii* in farm animals, but there are very limited studies concerning the presence of the bacterium in milk. The objectives of this study were (i) to determine the prevalence of *C. burnetii* in cows', goats', and ewes' bulk milk (BM) samples using PCR, (ii) to confirm positive results by DNA sequencing.

## Materials and methods

### Sample collection

A total of 150 BM samples (50 samples of each of cows', goats' and ewes' milk) were collected from 15 randomly selected dairy farms in Hatay province between January 2012 and July 2013. The samples were transported to the laboratory in a cooler with ice packs and stored at -20 °C until analysis.

### Bacterial strain

A *Coxiella burnetii* Nine Mile strain provided by University of Kirikkale, Turkey, was used as the positive control. The positive control used was a solution containing  $10^6$  coxiella-cells/mL. One milliliter of this solution was added to 1 mL of UHT (Ultra High Temperature treatment) milk and used as the positive control in each test for DNA extraction and PCR analysis. Nuclease free water (Promega, Madison, USA) was used as the negative control.

### PCR method

DNA extraction from milk samples was performed with DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. For the PCR assay, primers described by Hoover et al. (1992) were used. The PCR assay was based on the detection of the repetitive transposon-like gene of *C. burnetii* in this study.

The PCR was performed in a total volume of 50  $\mu$ L containing 1xPCR buffer (Sigma-Aldrich, St. Louis, USA), 2 mM MgCl<sub>2</sub> (Sigma-Aldrich), 200  $\mu$ M of each deoxynucleotide (Dr. Zeydanlı, Ankara, Turkey), 1 U Taq DNA polymerase (Sigma-Aldrich), 0.40  $\mu$ M of primers and 10  $\mu$ L of DNA.

The amplification protocol (Berri et al., 2000) was modified and carried out with the initial denaturation at 98 °C for 30 s, followed by 30 cycles, each consisting of 98 °C for 7 s, 60 °C for 20 s, 72 °C for 20 s, and final extension cycle of 7 min at 72 °C (Boeco, Hamburg, Germany). Amplified products were detected by agarose gel (1.5 %) electrophoresis (Clever) and visualized under UV transillumination (UVP, Upland, USA).

### DNA sequence analysis

The PCR amplification products were purified using an Agencourt Ampure purification kit (Beckman Coulter, Beverly, USA). A Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter) was used for sequence reaction. Sequence PCR products were purified using a Dye-Terminator removal kit (Agencourt CleanSEQ; Beckman Coulter). DNA sequences of the purified products were identified comparing to the DNA reference strain (*Coxiella burnetii* Nine Mile strain phase I, GenBank: U10529.1) in the GenBank.

## Results and discussion

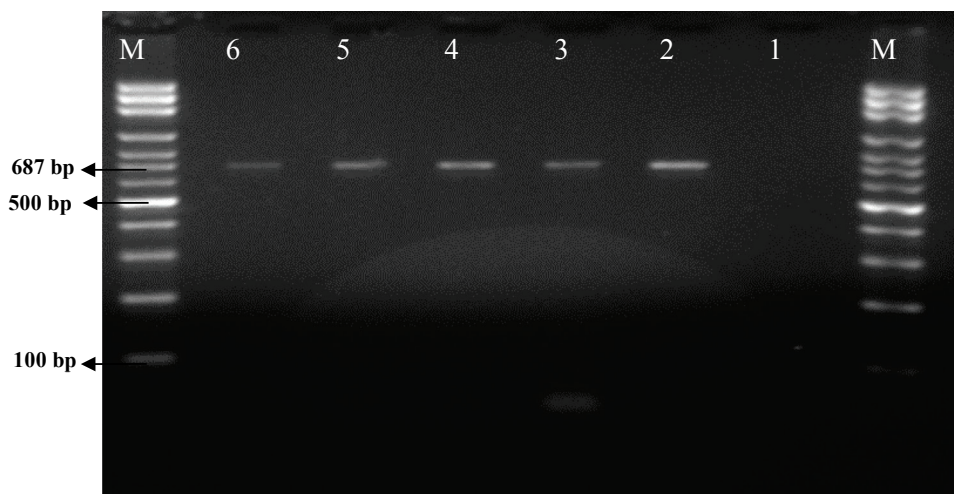
Of the 150 BM samples, 9 (6 %) were found positive for *C. burnetii* by PCR; five from cows' BM, two from ewes' BM, and two from goats' BM (Table 1). The PCR results are shown in Figure 1.

Positive results were confirmed by DNA sequencing. The sequencing results of *Coxiella* DNA extracted from cows', goats', and ewes' milk samples were consistent with the reference strain with 100 %, 99.7 %, 99.8 % homology, respectively.

Table 1. PCR results of *C. burnetii* in bulk milk (BM) samples

	No. of farms														
	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	4 <sup>th</sup>	5 <sup>th</sup>	6 <sup>th</sup>	7 <sup>th</sup>	8 <sup>th</sup>	9 <sup>th</sup>	10 <sup>th</sup>	11 <sup>th</sup>	12 <sup>th</sup>	13 <sup>th</sup>	14 <sup>th</sup>	15 <sup>th</sup>
No. of cows' BM	5	15	16	7	7										
No. of ewes' BM						5	5	4	6	15	15				
No. of goats' BM												25	11	4	10
No. of positive BM	0	0	0	0	5	2	0	0	0	0	0	0	2	0	0
Result of PCR	N	N	N	N	P	P	N	N	N	N	N	N	P	N	N

N, negative; P, positive



(M, 100 bp DNA marker; lane 1, negative control; lane 2, positive control (*Coxiella burnetii* Nine Mile strain); lanes 3-6, *C. burnetii* positive bulk milk samples)

Figure 1. Agarose gel electrophoresis of the amplification products of PCR assay

According to studies performed in different parts of Turkey, Dogru et al. (2010) investigated *C. burnetii* seroprevalences in farm animals and farmers and the presence of *C. burnetii* in milk samples. Milk samples of both seronegative and seropositive cows and sheep were analyzed by PCR, but *C. burnetii* was not detected in any of milk samples. There was a high seropositivity (90 %) in the farmers and it was reported that all the farmers were consuming dairy products made from raw milk of their animals.

Similar to the present study, milk samples from 400 sheep were analyzed and *C. burnetii* was found at a level of 3.5 % by immuno-magnetic-separation

(IMS)-PCR. It was indicated that positive samples were obtained from herds with a history of abortion and the abortions in these herds may have been due to Q fever (Öngör et al., 2004).

Parın and Kaya (2012) investigated a total of 600 blood samples from 200 cattle, 200 sheep and 200 goats for the presence of *C. burnetii* in Aydın province. According to ELISA method, the number of positive samples obtained from cattle, sheep and goats were 40 (20 %), 58 (29 %), 42 (21 %), respectively. By IFA method, 44 (22 %) cattle blood samples, 58 (29 %) sheep blood samples, and 46 (23 %) goats blood samples were found positive for *C. burnetii*. However, by using PCR technique

the number of positive samples obtained from cattle, sheep and goats were 96 (48 %), 72 (36 %), 46 (23 %), respectively. Results indicated that PCR was more sensitive than serological tests.

It was reported (Berri et al., 2000), that the trans-PCR showed a higher sensitivity and one *C. burnetii*-cell could be detected in 1 ml of milk by trans-PCR assay. A total of 359 bovine, 39 caprine, 81 ovine bulk milk samples were examined by Fretz et al. (2007) in Switzerland. *C. burnetii* was detected in 4.7 % of bovine bulk milk samples, while ovine and caprine bulk milk samples were all negative for the bacterium.

Kim et al. (2005) tested 316 bulk tank milk samples in the United States and the prevalence of *C. burnetii* was found 94.3 %. The sequencing results of the 687 bp PCR product were consistent with the reference strain with 100 % homology, similar to the current study.

In France, milk samples were taken from 242 dairy cows, among which 46 had aborted. By using the real time PCR technique, *C. burnetii* was detected in 59 (24.4 %) of 242 milk samples. Fifty (20.7 %) of the 242 feces samples and 46 (19.0 %) of the 242 vaginal mucus samples were also found positive. It was indicated that only milk may not give accurate information about *C. burnetii* shedding, because the bacteria can be shed through feces, vaginal mucus, and birth products (Guatteo et al., 2006). Rodolakis et al. (2007) used PCR to measure shedding of *C. burnetii* in milk, vaginal mucus and faeces of naturally infected sheep, goats and cattle. Ewes were found to shed more often *C. burnetii* in vaginal mucus and faeces, while cows and goats shed the bacteria more often in milk. Several animals shed the bacteria although they were seronegative by ELISA. Rodolakis et al. (2007) indicated the lack of sensitivity of the ELISA tests and the detection of antibodies in milk seems more sensitive than it is in serum. Rahimi (2010) reported that the prevalence of *C. burnetii* was 2 % in caprine bulk milk samples in Iran, which is very close to the values of the current study. In other study carried out by Rahimi et al. (2010), 13 of 210 (6.2 %) bovine milk samples were positive for *C. burnetii* by nested PCR. Ovine bulk milk samples were all negative and only one caprine bulk milk sample was positive for *C. burnetii*.

In southern Belgium, the high level of seropositivity (57.8 %) was found at dairy herds and of the 50 bulk tank milk samples from the selected herds, 15 (30 %) had a positive PCR, higher than our results (Czaplicki et al., 2012). Serological screening for *C. burnetii* infection was performed in Spain. A high seroprevalence and high bulk tank milk excretion ( $>10^4$  *C. burnetii*/mL) of the bacteria was found (Nogareda et al., 2012). In Denmark, higher rates (59 %) of antibodies against *C. burnetii* was found in bulk tank milk samples (Agger et al., 2010). Muskens et al. (2011) tested bulk milk samples of 12 herds in Netherlands and six samples (50 %) were positive in PCR and ten (83 %) were positive in ELISA.

Recently, PCR technique is a very useful method for the detection of *C. burnetii* in milk samples (Berri et al., 2000; Fretz et al., 2007; Öngör et al., 2004). A trans-PCR assay performed with primers based on a transposon-like repetitive region was proved to be highly specific and sensitive (Willems et al., 1994). Results of Guatteo et al. (2007) indicated that the proportion of milk samples which were positive for *C. burnetii* on the first test day after sampling day decreased by about one third when storing samples at +4 °C or -20 °C. The researchers suggest that PCR should be performed on the sampling day to minimize the risk of getting false-negative results.

## Conclusions

The results indicated that the presence of *C. burnetii* in BM samples may be a potential risk for public health.

In order to protect public health, food safety management systems such as Hazard Analysis Critical Control Point (HACCP), Good Manufacturing Practice (GMP) and Good Hygiene Practice (GHP) should be carried out in dairy farms.

*C. burnetii* is an important zoonotic agent, shedding into the environment through raw milk and dairy products. The fact that pasteurization time/temperature combinations has been established on requirements for destroying *C. burnetii* in milk. Unpasteurized milk is rarely consumed in Turkey, but some dairy products, especially cheese and butter, are locally produced from raw milk. Consequently,

more comprehensive studies are needed to better define the shedding of *C. burnetii* in milk in Turkey.

*Detekcija Coxiella burnetii  
u skupnim uzorcima kravljeg, kozjeg  
i ovčjeg mlijeka pomoću lančane reakcije  
polimeraze (PCR)*

### Sažetak

Q-groznica je široko rasprostranjena zoonoza koju uzrokuje obligatno unutarstanična bakterija *Coxiella burnetii*. Ovom studijom planirano je utvrditi prevalenciju *C. burnetii* u skupnim uzorcima kravljeg, kozjeg i ovčjeg mlijeka pomoću PCR i potvrditi pozitivne rezultate sekvenciranjem DNA. Analizirano je ukupno 150 uzoraka (50 uzoraka kravljeg, kozjeg i ovčjeg), sa 15 nasumično odabranih gospodarstava u pokrajini Hatay. Skupni uzorci uzeti su u razdoblju između siječnja 2012. i srpnja 2013. Bakterijska DNA izolirana je direktno iz mlijeka. Devet je uzoraka (6 %) bilo PCR pozitivno (pet uzoraka kravljeg mlijeka i po dva kozjeg i ovčjeg). Bakterijska DNA utvrđena je u 3 od 15 (20 %) mliječnih farmi. Pozitivni rezultati potvrđeni su sekvenciranjem bakterijske DNA, jer je utvrđena vrlo visoka (100 %, 99,7 %, 99,8 %) homolognost sekvenci sa referentnim sojem *Coxiella burnetii*, stoga je nužno provođenje higijenskih mjera da bi se osigurala sigurnost hrane i zdravlje ljudi.

*Ključne riječi:* skupno mlijeko, *Coxiella*, lančana reakcija polimeraze, analiza sekvenci

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