Identification of *Coxiella burnetii* by touch-down PCR assay in unpasteurized milk and dairy products in North - East of Iran

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Abstract:

BACKGROUND: *Coxiella burnetii* is the causative agent of the zoonotic disease Q fever, and ruminants being considered as the main source for human infection. Although the main route of infection in human is inhalation of contaminated aerosols, oral transmission by contaminated raw milk or unpasteurized dairy products is also a possible route of infection. Raw milk or dairy products produced from unpasteurized milk may contain virulent *C. burnetii*. **OBJECTIVES:** This study aimed to determine the contamination rate of milk and unpasteurized dairy products with *C. burnetii*. **METHODS:** Touch-down PCR was used to examine the presence of *C. burnetii* on 147 dairy product samples collected from local traditional and commercial markets in Mashhad-Khorasan Razavi province- Iran. **RESULTS:** 2 of 28 (7.14%) cheese samples, 2 of 26 (7.69%) yoghurt samples, 8 of 23 (34.78%) sheep milk samples, and 2 of 60 (3.33%) cow milk samples were found to be positive for *C. burnetii* DNA. However, 10 goat milk samples were found to be negative. **CONCLUSIONS:** The results of this study indicate that the clinically healthy dairy livestock and their dairy products are important sources of *C. burnetii* infection.

Introduction

Coxiella burnetii is strictly intracellular Gram-negative bacteria, a short (0.3 to 1.0 μm) and pleomorphic rod organism. It is the causative agent of a zoonotic disease that occurs in human (Q fever) and animals (coxiellosis). *C. burnetii* is extremely resistant to heat, pressure, and chemical stress and can survive for months in stressful environments (Rahimi et al., 2010). This organism is also highly infectious. In experimental conditions, only one organism is required to produce infection (Ormsbee et al., 1978).

Q fever is a worldwide zoonotic disease and has been reported from most countries except New Zealand (Fournier et al., 1998). Recent studies show that Q fever is a considerable public health problem in many countries, especially people who are in direct contact with domestic animals. People who are in contact with animals, including veterinarians, farm workers, slaughterhouse workers, and laboratory personnel working with infected animals are at higher risk (Maurin and Raoult, 1999; Kirkan et al., 2008).

Cattle, sheep, and goats are the major reservoirs of *C. burnetii*. Also a wide variety of other animals can be infected with *C. burnetii*, including dogs, cats, non-human primates, wild rodents, small mammals, big game wildlife, non-mammalian animals, including reptiles, amphibians, domesticated and wild birds, fish, and ticks (Parker et al., 2006). Ticks are normally the primary reservoir of these bacteria and also the distributer of bacteria in wild and

domestic animals (Abbasi et al., 2011).

The organism is shed via urine, feces, and milk of infected animals and has a particularly high concentration during parturition. Shedding into the environment occurs mainly by birth products, particularly the placenta. In the chronic phase, the uterus and mammary glands are primary sites of infection for *C. burnetii* (Maurin and Raoult, 1999; Kim et al., 2005). The main transmission route of *C. burnetii* for human is respiratory aerosols or dust contaminated with birth fluid, placenta, urine, and feces of infected animals.

Although animals are often the main source of infection for human, they do not show the coxiellosis symptoms clearly, except in cases of abortion in the last weeks of pregnancy, infertility (which has been reported in cattle and its occurrence has not been reported in sheep), metritis, mastitis, and stillbirth. Abortion occurs in sheep and goat, but less frequently in cattle (Barlow et al., 2008; Kirkan et al., 2008). In human, Q fever is most often asymptomatic, but acute disease (mainly a limited flu-like illness, pneumonia or hepatitis) or chronic disease (chronic fatigue syndrome or endocarditis) can occur (Fournier et al., 1998).

The gastrointestinal route (consumption of row milk and unpasteurized dairy products) is of minor importance (Rahimi et al., 2010). It has been reported that up to 105 cfu ml-1 coxiellae can be shed in bovine milk during several lactation periods (Biberstein et al., 1974). Therefore, a specific and sensitive diagnostic system is necessary to detect even small numbers of this microorganism.

In previous studies, serological tests were the main way to determine the prevalence of *C. burnetii* infection (Berri et al., 2000), however, it may indicate a history of previous exposure to *C. burnetii*. Cell culture is a sensitive method for detection of *C. burnetii*, but this method is time-consuming. Capture enzyme-linked immunosorbent assay (ELISA) method is faster than cell culture; however, considering the low level of shedding and the minimum infectious dose of *C. burnetii*, the detection limit is not completely satisfactory (Lorenz et al., 1998). Polymerase chain reaction (PCR) is a highly sensitive and specific detection method that has been used for screening (Kim et al., 2005; Ongor et al., 2004) and determining the presence of the bacteria in milk, feces, or vaginal swabs (Berri et al., 2000).

The objective of the present study was to determine the presence of *C. burnetii* in raw milk and dairy products that are made from unpasteurized milk, in Mashhad using a touchdown PCR assay.

Materials and Methods

Sampling: From January to May 2012, a total of 147 samples of raw milk or dairy product which were prepared from unpasteurized milk were collected from dairy farms and retail stores in different areas in Mashhad city, Khorasan-Razavi province of Iran. The samples included 10 goat' raw milk, 23 sheep's raw milk, 60 cow's raw milk, 28 cheese samples (100 gram each) which were made from sheep milk and 26 yoghurt samples with the same origin. Samples were collected aseptically and placed in a cooler box with ice packs and immediately transferred to the laboratory. The samples were processed within an hour of collection or stored at -20°C until use.

DNA extraction from raw milk: Bacterial DNA from milk samples were extracted by centrifuging and removing the cream and milk layers as described previously by Berri et al., (2003) with some modifications. Briefly, 50ml of each milk sample was transferred to the 50 ml falcon tube and centrifuged 3 times at 3000 g for 10 minutes. Each time the supernatant was discarded and replaced by phosphate buffered saline (PBS). Purification of DNA was achieved using a genomic DNA extraction kit (Bioneer, South Korea) according to the manufacturer's instruction, and the total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001).

DNA extraction from cheese and yoghurt: Briefly, 5g of cheese or 5ml of yoghurt were transferred to the stomacher bag, then 45 ml of the diluent (0.5% w/v sodium chloride, 1% w/v casitone, 2% w/v sodium citrate) were added and the bags were squeezed manually to dispense the diluent. The bags were placed into stomacher and stomached for 5 min, then heated at 50°C for 2 h; this step was repeated 4 times (Hirai et al., 2012). The rest of the process was the same as raw milk processing.

DNA amplification (trans-PCR): In this study, a polymerase chain reaction (PCR) assay targeting a transposon-like repetitive region of the bacterial

genome (IS1111 gene) was used to detect *C. burnetii*. Trans-1 and trans-2 primers with the following sequence were used from the published data. Trans-1 (5'-TAT GTA TCC ACC GTA GCCAGT C-3') and trans-2 (5'-CCC AAC AACACC TCC TTA TTC-3') (Hoover et al., 1992). Primers were synthesized by Bioneer Co. (South Korea). These primers amplify a 687-bp fragment of the target sequence. PCR assay was performed as described previously (Vaidya et al., 2008). The PCR mixture (25µL) included 2.5µL of 10×PCR buffer (100 mM Tris-HCl buffer, pH 8.3, 500 mM KCl, 15 mM MgCl2, and 0.01% gelatin), 200 µM deoxynucleoside triphosphate mix, 2µM of each primers, 0.3 U of Taq DNA polymerase, 3µL of template DNA, and high pure double sterilized water to make up the reaction mixture volume. The amplification was performed in a personal thermo-cycler (TECHNE TC- 5 UK). The cycling denaturation of DNA at 95°C for 2 min, followed by five cycles at 94°C for 30s, 66 to 61°C (the temperature was decreased by 1°C between consecutive steps) for 1 min, and 72°C for 1 min. These cycles were followed by 35 cycles consisting of 94°C for 30 s, 61°C for 30 s, and 72°C for 1 min and then a final extension step of 10 min at 72°C (Hoover et al., 1992).

After electrophoresis of amplicons in agarose gel and staining with ethidium bromide at concentration of 0.5 mg mL-1, they visualized under UV illumination.

After confirmation of the first positive PCR product as *C. burnetii* by sequence analysis, it was used as positive control, and for negative control deionized distilled water was used.

Sequence analysis: The first positive PCR product was purified using the Roche purification kit (Roche Molecular Biochemicals, Mannheim, Germany) and submitted for automated sequencing in both directions at the Eurofins MWG Operon (Martinsried, Germany) using PCR primers as sequencing primers. Nucleotide and predicted amino acid sequence data were aligned with the clustal alignment algorithm. Phylogenetic analysis based on nucleotide sequences was conducted using a distance method, unweighted pair group with arithmetic mean, by calculating boots trap values for 1000 replicates in CLC main Workbench Package Version 5 (CLC Bio, Aarhus, Denmark).

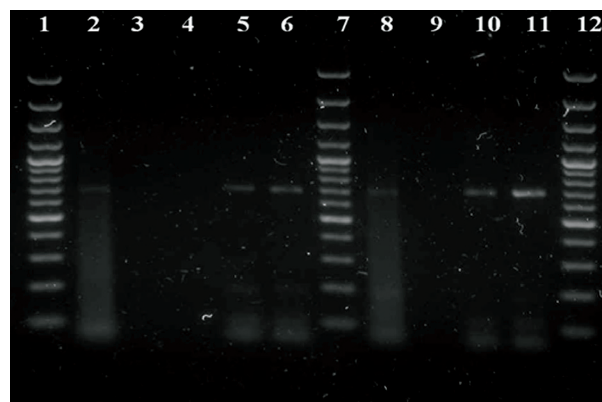

Figure 1. Detection of *Coxiella burnetii* in milk and dairy products using touch-down PCR assay, amplifying a 687 bp segment of the IS1111 gene: Lanes 1, 7, 12 100bp markers. Lane 2 positive control. Lane 3 negative control (DW). Lanes 5, 6 positive samples for *C. burnetii* in bovine milk samples. Lanes 8, 10, 11 positive samples for *C. burnetii* in yoghurt samples.

Results

The presence of *C. burnetii* was evaluated in sheep, goat and cow raw milk and also cheese and yoghurt samples which were made from sheep milk. After the DNA extraction, touch-down PCR assay targeting the IS1111 gene of the organism by Trans-1 and trans-2 primers resulted in 2 of 28 cheese samples (7.14%), 2 of 26 yoghurt samples (7.69%), 8 of 23 sheep milk samples (34.78%), and 2 of 60 (3.33%) bovine milk samples as positive for *C. burnetii*, whereas all 10 goat milk samples were detected as negative. A sample photograph of gel electrophoresis is shown in Figure 1.

For positive control, the 687 base pairs of the amplified gene fragment were successfully sequenced from the first PCR-positive sample and by comparing to the published sequences of *C. burnetti* in Gene bank. No differences in nucleotide and deduced amino acid were found.

Discussion

The most commonly identified sources of human infections with *C. burnetti* are farm animals such as cattle, goats, and sheep. Mammals can shed *C. burnetii* in milk, and thus consumption of raw milk and dairy products which are made from un-pasteurized milk could be a source of infection

(Fournier et al., 1998).

In order to identify *C. burnetii* in milk and dairy products, the PCR method is a safe and useful method, whereas conventional isolation of *C. burnetii* is hazardous, difficult, and time-consuming; besides, the isolation of this microorganism must be performed in biosafety-level 3 laboratories (Barlow et al., 2008; Khalili et al., 2011; Arricau-Bouvery and Rodolakis, 2005). Although this method could not determine the viability of the organisms in raw milk and dairy products, some studies have compared results of PCR detection of *C. burnetii* in milk with bacterial viability assay by mouse inoculation (Rahimi et al., 2010; Hoover et al., 1992). While these studies demonstrated PCR positive milk samples contained viable organisms, additional studies are needed to determine how PCR based detection relates to the potential infectiousness of *C. burnetii* in milk samples, and the sensitivity and specificity of PCR relative to inoculation or antigen detection assays (Barlow et al., 2008). Only a few studies have described the presence of *C. burnetii* in dairy products such as cheese (Hirai et al., 2012). Furthermore, there have been no reports on detection of *C. burnetii* in yoghurt by PCR assay and this study is the first report in detection of DNA *C. burnetii* in yoghurt.

In this study, for detection of *C. burnetii* in raw milk and dairy produts, PCR assay was used for targeting the repetitive transposon-like region of *C. burnetii* (Trans-PCR). The efficiency of the method for detection of Coxiella in milk samples was further improved and one *C. burnetii*-cell could be detected in 1 ml of milk (Berri et al., 2000) and it has been proved that trans-PCR has a high sensitivity and specificity (Kim et al., 2005; Barlow et al., 2008; Kirkan et al., 2008; Berri et al., 2009).

In order to prepare the PCR mixture and excluding the PCR inhibitors which might be present in raw milk and dairy products, samples were centrifuged three times and each time the pellet were resuspended in PBS. It has been reported that, the detection limit for *C. burnetii* in PBS was 10-fold higher than that in milk (Muramatsu et al., 1997).

According to these findings, DNA sequence of *C. burnetii* has been detected in 3.33% of cow milk, 34.78% of ovine milk, 7.14% of cheese and 7.69% of yoghurt samples. The size of this survey does not allow any statistical statement, and possibly because of our sample size, goat's milk samples were detected as negative. These data only show the shedding of *C. burnetii* through bovine and ovine milk and consequently the presence of their DNA sequence in milk products.

Other studies have reported a different range of the presence of this microorganism in milk. 1.8% in goat milk and 0% in Iranian sheep milk (Rahimi et al., 2010), 3.5% of ovine milk samples from Turkey (Maurin and Raoult, 1999) and 0% of goat and sheep milk from Switzerland (Kim et al., 2005; Fretz et al., 2007), whereas 83.8% of cow milk from France (Berri et al., 2000), 53.7% from Japan (Maurin and Raoult 1999) and 14.3% from Italy (Ongor et al., 2004) were positive for *C. burnetii*. However, for the presence of this microorganism in cheese the only report is 17.1% from Japan (Hirai et al., 2012).

It should be considered that *C. burnetii* might shed by other routes such as vaginal mucus, feces, urine, placenta, or birth fluids. Testing an animal based on only milk sample can lead to misclassify the status of the animal. Sheep shed *C. burnetii* mainly in feces and vaginal mucus; whereas, cow shed *C. burnetii* mainly in milk and goat excrete *C. burnetii* in their vaginal discharges, feces, and milk. Furthermore, the infected animals may not persistently shed this microorganism (Guatteo et al., 2007).

The results of this study indicate a potential risk to the public health associated with the presence of *C. burnetii* in raw milk and unpasteurized dairy products in this area of Iran, which may be viable and infectious.

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شناسایی کوکسیلا بورنتی با استفاده از واکنش زنجیره‌ای پلیمراز به روش تاچ داون در شیر و فرآورده‌های لبنی غیر پاستوریزه

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چکیده

زمینه مطالعه: کوکسیلا بورنتی عامل بیماری مشترک تب‌کیو است و نشخوارکنندگان منبع اصلی عفونت انسانی محسوب می‌شوند. اگرچه راه اصلی ایجاد عفونت در انسان از طریق استنشاق آئروسل‌های آلوده است، اما انتقال خوراکی از طریق مصرف شیر خام و فرآورده‌های لبنی آلوده نیز از راه‌های احتمالی ایجاد عفونت محسوب می‌شود. شیر خام و فرآورده‌های لبنی تهیه شده از شیر غیر پاستوریزه ممکن است حاوی کوکسیلا بورنتی بیماری‌زا باشد. **هدف:** این مطالعه با هدف تعیین میزان آلودگی شیر و فرآورده‌های لبنی به کوکسیلا بورنتی انجام شد. **روش کار:** در این مطالعه برای آزمایش حضور کوکسیلا بورنتی در ۱۴۷ نمونه فرآوردهٔ لبنی جمع‌آوری شده از فروشگاه‌های عرضهٔ محصولات سنتی و صنعتی در شهر مشهد، استان خراسان رضوی، واکنش زنجیره‌ای پلیمراز به روش تاچ‌داون استفاده شد. **نتایج:** دو نمونه از ۲۸ نمونه پنیر (۷/۱۴٪)، دو نمونه از ۲۶ نمونه ماست (۷/۶۹٪)، ۸ نمونه از ۲۳ نمونه شیر گوسفند (۳۴/۷۸٪) و دو نمونه از ۶۰ نمونه شیر گاو (۳/۳۳٪) از نظر کوکسیلا بورنتی مثبت بودند. در ۱۰ نمونه شیر بز مورد آزمایش کوکسیلا بورنتی یافت نشد. **نتیجه‌گیری نهایی:** نتایج به‌دست‌آمده از این مطالعه نشان می‌دهد که دام‌های سالم از نظر بالینی و فرآورده‌های لبنی آنها از منابع مهم آلودگی به کوکسیلا بورنتی محسوب می‌شوند.

واژه‌های کلیدی: کوکسیلا بورنتی، شیر، واکنش زنجیره‌ای پلیمراز به روش تاچ داون، فرآورده‌های لبنی

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