Molecular investigation of *Coxiella burnetii* infections in aborted sheep in eastern Turkey

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Summary

Q fever is a zoonotic disease that occurs worldwide and is caused by the obligate intracellular bacterium *Coxiella burnetii*. The aim of this study was to investigate the presence of *C. burnetii* infection in aborted sheep in eastern Turkey using PCR. A total of 200 fetuses were collected from aborted sheep belonging to 200 herds in different locations in the eastern part of Turkey. Foetal organ samples such as liver, spleen, lung and stomach were taken and the DNA was purified from two hundred pooled samples. PCR analysis of *C. burnetii* presence in infected organs was performed, and 4 samples (2%) were found positive. In addition, the pooled organ suspensions were inoculated to embryonated chicken eggs, and PCR analysis of yolk sacs showed *C. burnetii* DNA in 5 samples (2.5%). This study shows that *C. burnetii* infection has an important role in sheep abortions in eastern Anatolia region.

Key words: Abortion, Eastern Turkey, PCR, Q fever, Sheep

Introduction

Coxiella burneti is a small gram negative bacterium that dwells in host cells and varies between bacillus and coccus forms. *Coxiella burnetii* is an obligate intracellular pathogen that causes Q fever disease in several animal species and in humans (Raoult and Marrie, 1995; Maurin and Raoult, 1999). The infection has been observed in a wide range of animals, particularly pets and livestock (Lang, 1990). Cattle, sheep and goats are primary *C. burnetii* reservoir hosts (Arricau-Bouvery and Rodolakis, 2005). Although abortion is related to *C. burnetii* in sheep and goats, it is reported not to cause any clinical diseases in these animals (Lang, 1990). The organism is eliminated in milk, urine and faeces of the infected animals (Berri *et al*., 2000; Vaidya *et al*., 2010; Pritchard *et al*., 2011; Muskens *et al*., 2012). It is reported that living *C. burnetii* microorganisms are present for 150 days following birth or abortion and that ruminants release them in milk for up to 32 months (Rodolakis *et al*., 2007). It has been reported that the pathogen can infect humans and animals by inhalation of contaminated aerosols of urine, faeces, placenta and by consumption of raw milk and dairy products as well (Norlander, 2000).

Q fever diagnosis is performed using smear staining, serology, PCR and direct isolation of *C. burnetii* (Rousset *et al*., 2007; Anderson *et al*., 2013). However, serological testing in domestic ruminants showed only Q fever occurrence in the herd, and does not allow bacteria shedding to be detected by infected animals. In addition, *C. burnetii* is difficult to isolate, time consuming, and requires biosafety level 3 laboratory (OIE, 2010).

There are few studies on the prevalence of the agent in pregnant animals or aborted foetuses in Turkey using PCR technique to detect the agent (Öngör *et al*., 2004; Akgün *et al*., 2006; Kırkan *et al*., 2008). Kırkan *et al*. (2008) detected *C. burnetii* DNA in 6 (4.3%) of the serum samples collected from a total of 138 blood samples from cattle in farms. Öngör *et al*. (2004) when examining the presence of *C. burnetii* in 400 sheep milk samples via IMS-PCR, obtained positive results in 14 (3.5%) samples.

This study was performed to determine the extent of *C. abortus* infection in aborting sheep in eastern Turkey, and to evaluate the significance of culture and PCR assays for detection of *C. burnetii* infections in sheep.

Materials and Methods

Sample collection for *C. burnetii*

Sheep abortion cases were observed in 6 different locations (Elazığ, Malatya, Tunceli, Bingöl, Bitlis and Muş) in the eastern part of Turkey, and a total of 200 aborted foetuses were collected from 200 herds. Liver, spleen, lung and stomach internal organs were taken for each foetus. The samples taken from animals that underwent abortion were stored at 4°C if they were to be processed within 24 h or at -70°C when they were processed later.

Organ samples processing

Organ samples from the foetus (liver, spleen, lung and stomach) were transferred to the laboratory within sterile containers for DNA isolation. Two hundred pooled samples from foetal tissues were prepared. For this, foetal tissues were cut into small pieces, homogenized in PBS buffer containing 1000 units/ml of penicillin (Pharmacia-Upjohn) and 1000 units/ml streptomycin sulfate and then incubated in 2% trypsin (Difco) at 37°C for 3 h. Homogenized tissue was then centrifuged at 6000 × g for 10 min, the supernatant was taken and filtered through cellulose acetate filters with 0.45 µm diameter. After that, the homogenized tissue was resuspended in fetal bovine serum (FBS, GibcoBRL) containing 10% dimethylsulphoxide (DMSO, Sigma) and stored at -20°C by aliquoting 1 ml samples into cryovials. For the agent identification, the suspension of 200 foetal organ samples was inoculated into yolks of 6-7 day old chicken eggs embryos, and incubated at 37°C for 10-15 days. After the fifth day, eggs with embryo death were opened up and slides were prepared from egg yolk membranes. Prepared slides were analyzed by staining using the Gimenez method. Stained smears of the yolk sacs were examined to ensure the absence of bacterial contamination and to demonstrate the presence of *C. burnetii* (Maurin and Raoult, 1999). Obtained isolates were identified using PCR assay as described in the next section.

DNA isolation and PCR amplification

DNA purification from both tissue samples and infected embyronated egg yolks was performed using the QIAamp DNA kit (Qiagen Courtaboeuf, France) following the manufacturer's recommendations. Two different pairs of primers were used in order to compare the sensitivity of PCR. The first pair of primers targeted the gene that encodes superoxide dismutase enzyme of *C. burnetii* (257-bp band) (Stein and Raoult, 1992): C.B.-1: 5´- ACT CAA CGC ACT GGA ACC GC-3´ and C.B.-2: 5´- TAG CTG AAG CCA ATT CGC C-3´. The second set targeted a species-specific transposon repetitive region of *C. burnetii* (687-bp band) (Houver *et al*., 1992): Trans 1: 5´- TGG TAT TCT TGC CGA TGA C-3´; Trans 2: 5´- GAT CGT AAC TGC TTA ATA AAC CG-3´.

The Trans-PCR thermal program was carried out under the following conditions: initial denaturation at 95°C for 2 min followed by 5 cycles and then for 40 cycles consisting of denaturation at 94°C for 30 s, annealing at, 61°C for 30 s and extension at 72°C for 1 min, then a final extension at 72°C for 10 min (Vaidya *et al*., 2010).

The PCR amplification program for CB1-2 primers was 30 cycles consisting of denaturation at 95°C for 20 s, annealing at 50°C for 1 min and extension at 72°C for 2 min (Stein and Raoult, 1992). Amplified PCR products were run on a 1.5% agarose gel for electrophoresis under 100 volts for 1 h. Following electrophoresis, the gel was stained using ethidium bromide (0.5 µg/ml) and results were evaluated by UV transillumination. The obtained 687-bp band when using the Trans primers was assessed as positive for *C. burnetii*. Positive control DNA and water samples were included in all amplifications. This band is obtained using a positive control DNA purified from *C. burnetii* isolated from aborted placenta of sheep and kindly provided by the INRA Research Institute (Institut National de la Recherche Agronomique, France). Nuclease free water (Promega, Madison, USA) was used as the negative control.

Results

Comparison of CB1/CB2 and Trans-PCR sensitivity

Four clinical samples (pooled samples) were tested using both CB1/CB2 and Trans-PCR, and all tested samples gave a 687-bp PCR product with trans primers. No PCR product was observed using distilled water instead of DNA as template (Fig. 1). However, CB1-CB2 primers did not allow *C. burnetii* detection in clinical samples. The selected trans primers were found to be more sensitive than CB1-CB2.

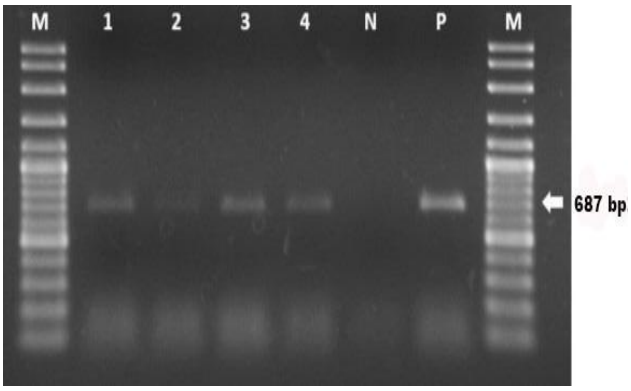

Fig. 1: Agarose gel staining with ethidium bromide of *C. burnetii* PCR products. M: 100 bp DNA ladder. Lines 1-4: Positive samples, N: Negative control, and P: Positive control DNA

Detection of *C. burnetii* in clinical samples

A total of 200 biological samples of foetal tissues (each of livers, spleens, lungs and stomachs) of aborted sheep were tested using Trans-PCR. Agarose gel staining showed C. *burnetii* DNA in 4 samples (2%) (Fig. 1).

C. burnetii isolation

All clinical samples were inoculated into 6- to 7-day-old embryonated chicken eggs. The mortality of embryos in the inoculated eggs and the demonstration of typical elementary bodies (EB_s) determinative of *C. burnetii* infection in the yolk sac membranes (YSM) were observed in five samples. During the examination of smears from the YSM of eggs the characteristics of inclusions or EB_s were observed. When the smears were stained with Gimenez's stain, it appeared as red intracytoplasmic bodies against a green background of cytoplasm. Stained smears of yolk sac wall were examined to ensure the absence of bacterial

contamination and to establish presence of *C. burnetii*. Typical inclusions or EB_s determinative of *C. burnetii* infection in YSM were observed in 5 samples (2.5%). The same samples were found positive for *C. burnetii* by PCR from embryonated egg yolk sacs.

Discussion

Q fever is known to be endemic in humans and livestock and to be widespread across Turkey (Çetinkaya *et al*., 2000; Kılıç *et al*., 2005; Kennerman *et al*., 2010; Parın and Kaya, 2012), and abortions in sheep caused by infections lead to great economic loss.

This study was carried to improve our knowledge regarding Q fever prevalence among ruminant's herd in Turkey. So first the sensitivity of two PCR assays was compared to detect *C. burnetii* DNA. Two different sets of primers were used in order to evaluate the sensitivity of the PCR (CB1-CB2 primers and Trans 1-Trans 2 primers). Among these sets, Trans 1 and Trans 2 primers that amplify the species-specific transposon-repetitive region of *C. burnetii* have been reported to successfully identify *C. burnetii* in different materials (Berri *et al*., 2000; Nourollahi and Khalili, 2011; Kargar *et al*., 2014). Several Q fever investigations found Trans-PCR to be highly specific and sensitive for the direct detection of *C. burnetii* in genital swabs, milk and faecal samples from ewes (Willems *et al*., 1994; Berri *et al*., 2000; Kim *et al*., 2005; Vaidya *et al*., 2008). We compared PCR assays using Trans 1-Trans 2 and CB1-CB2 primers and found Trans-PCR more sensitive and specific. The PCR using Trans 1 and Trans 2 primers was found to be a reliable technique for the detection of *C. burnetii* in sheep abortion materials. The efficacy of Trans-PCR is attributed to the targeted region which exists in at least 19 copies in the *C. burnetii* Nine Mile, phase 1, genome (Berri *et al*., 2000; Hoover *et al*., 2002). However, the PCR positive samples detected in our study were very low compared to the data that were published regarding PCR investigation of Q fever in Turkey. This low rate of PCR positive foetal tissues might be due to the low amount of agent present in the samples or leading factors that inhibit PCR in foetal tissue samples.

Isolation of *C. burnetii* is not performed routinely for diagnostic purposes in veterinary medicine, because it is time consuming, hazardous, expensive and requires extensive laboratory. But *C. burnetii* isolation in embryonated yolk sacs is easier than isolation in tissue cultures; the growth and multiplication of the intracellular bacterium leads to death of the embryo, usually within 14 day postinoculation (Maurin and Raoult, 1999). The Gimenez method is usually used to stain *C. burnetii* inlaboratory cultures or clinical specimens. In the present sutdy, *C. burnetii* was identified in 5 of the 200 sheep aborted foetuses (2.5%) using culture from embryonated egg yolk sacs inoculated with foetal organ suspensions. All the positive results of 2.5% (5/200) obtained in the present study with Gimenez staining were confirmed by PCR.

However, the rate in our finding (2.5%) when compared the findings of other researchers was positive at low levels (Akgün *et al*., 2006; Parın and Kaya, 2012; Günaydın *et al*., 2015). This low rate of *C. burnetii* presence in sheep foetus may be due to the sanitary precautions, seasonal pattern, good hygiene on farm or geographic differences factors. The study is important since it helps in the identification of *C. burnetii*, especially in epidemiological studies, which can lead to prevention of infection of humans that are in close contact with the animals. It was demonstrated that *C. burnetii* can be rapidly identified in clinical samples sent to the laboratory using commercially available PCR kits in routine diagnoses and epidemiological studies. In addition, Trans-PCR assay was a better diagnostic method to investigate the presence of *C. burnetii* infection in aborted sheep. This study can be a guide for other molecular studies aiming to prevent the spread of the zoonotic pathogen *C. burnetii* in animal products.

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Conflict of interest

The authors declare that they have no conflict of interest.

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