

PCR Detection of *Coxiella burnetii* in Fetal Abomasal Contents of Ruminants

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Abstract

This study was carried out on abomasal contents of aborted fetuses of 102 cattles, 45 sheep and 5 goats sent to the laboratory from Central Anatolia, and Black Sea Region of Turkey to investigate the presence *Coxiella burnetii* (*C. burnetii*) by trans-PCR. Out of 152 abomasal contents, 11 (7.23%) were detected to contain *C. burnetii* DNA. Four (3.92%) out of 102 cattle, 5 (11.11%) out of 45 sheep, and 2 (40%) out of 5 goat abomasal contents harbored *C. burnetii* DNA. As a result, PCR detection of *C. burnetii* DNA in fetal abomasum contents is a significant finding of a possible *C. burnetii* related abortion.

Keywords: *Coxiella burnetii*, Fetal abomasal contents, Trans-PCR

Ruminantların Fötal Abomasal İçeriklerinde *Coxiella burnetii*'nin PCR ile Tespiti

Özet

Bu çalışma, İç Anadolu ve Karadeniz Bölgesi'nden laboratuvara gönderilen 102 sığır, 45 koyun ve 5 keçi atık fötüs mide içeriğinde trans-PCR ile *Coxiella burnetii* (*C. burnetii*) varlığını araştırmak için yapıldı. Yüz elli iki abomasal içeriğin, 11'inin (%7.23) *C. burnetii* DNA'sı içerdiği tespit edildi. 102 sığırın 4 (%3.92)'ünün, 45 koyunun 5 (%11.11)'inin ve 5 keçinin 2 (%40)'sinin abomasal içeriklerinde *C. burnetii* DNA'sı tespit edildi. Sonuç olarak, fetal abomasal içeriklerde PCR ile *C. burnetii* DNA'sı tespit edilmesinin, *C. burnetii* ilişkili abortlar için önemli bir bulgu olduğu düşünüldü.

Anahtar sözcükler: *Coxiella burnetii*, Fötal abomasal içerik, Trans-PCR

INTRODUCTION

Q fever is a highly contagious zoonotic disease caused by *Coxiella burnetii*, a Gram-negative obligate intracellular bacterium, which has an ability to survive in phagolysosomes and resist against unfavorable environmental conditions ^[1]. Cattle, sheep, and goat are the primary reservoirs for *C. burnetii* ^[2]. *C. burnetii* can infect broad spectrum of susceptible hosts including wildlife, and even non-mammalian species comprising ticks, birds, and reptiles. Any infected animal has the potential to transmit the pathogen via bacterial shedding in their body secretions. Transmission occurs mainly through the inhalation of aerosols formed during parturition or at slaughter ^[3].

Routine diagnosis of Q fever is usually established by serological tests such as, immunofluorescence, complement fixation and enzyme-linked immunosorbent assay (ELISA) ^[4]. However, seroconversion typically occurs 7-15 days after symptoms appear. For a definitive diagnosis in the early stages of acute Q fever, serologic testing in combination with PCR is recommended ^[5]. Isolation of *C. burnetii* is restricted to specialized laboratories and not preferred in veterinary medicine, since *C. burnetii* does not grow on standard laboratory bacteriological media and its isolation is time consuming, laborious, hazardous to perform and requiring biosafety level 3 laboratories ^[6]. PCR-based diagnostic assays have been developed for the detection of *C. burnetii* DNA and these have been used primarily for clinical samples ^[7,8]. Recently, other types



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of PCR assays, like nested PCR [9,10] and quantitative PCR (qPCR) [11,12] have been developed, and used sometimes in combination with high-throughput capabilities [13].

Several PCR-based methods have been developed targeting the isocitrate dehydrogenase gene; *icd* [14], the superoxide dismutase gene; *sod* [15], the outer membrane protein-coding gene; *com1* (10), and a transposon-like repetitive region; *IS1111* [16]. A PCR performed with primers based on a repetitive, transposon-like element (Trans PCR) [16] has proved to be highly specific and sensitive for the laboratory diagnosis of *C. burnetii* infections, as even very few copies of a specific DNA sequence can be detected.

The purpose of this study was to investigate *C. burnetii* in the abomasal contents of aborted fetuses of ovine, caprine and bovine origin by conventional PCR (Trans-PCR) targeting transposon-like repetitive element.

MATERIAL and METHODS

Samples

Between the years 2009-2011, a total of 152 abomasal contents of the aborted fetuses consisting of 102 cattles, 45 sheep, 5 goats were sent to the laboratory under sterile conditions and cold chain. The distribution of the abomasal contents sent to the laboratory among the ruminants and provinces were mentioned in *Table 1*. At the time of arrival, DNAs were extracted from all samples.

DNA Extraction

DNA extracted from positive strain containing the gene coding phase II antigen was kindly obtained from Department of Microbiology, Faculty of Veterinary Medicine, Fırat University, and, DNAs from all abomasal contents were extracted by commercial DNA isolation kit (DNeasy Tissue Kit, Qiagen, Germany) according to the manufacturer's instructions. DNAs were stored at -20°C until used.

Primers

Trans-1 and trans-2 primers, specific to the *IS1111* fragment, a transposon-like repetitive region were targeted for the detection of *C. burnetii* by Trans-PCR. Primers as previously described by Hoover et al. [16] consisted of the following sequences: Trans 1; 5'-TAT GTA TCC ACC GTA GCC AGT C-3' and Trans-2; 5'-CCC AAC AAC ACC TCC TTA TTC-3'. Expected amplicon size was 687 bp (*Fig. 1*).

Trans PCR

Each reaction had a volume of 25 µl including, 22 µl reaction mixture containing 2.5 µl 10× PCR buffer (without MgCl₂), 0.5 µl dNTP (10 mM), 1.5 µl MgCl₂ (25 mM), a 1 µl of each primer (10 pmol/µl), 0.25 µl Taq DNA polymerase (5 U/ µl) (Fermantas, Vilnius, Lithuania), 15.25 µl deionized

Table 1. The distribution of the abomasal contents sent to the laboratory among ruminants and provinces

Tablo 1. Laboratuvara gönderilen mide içeriklerinin ruminantlar ve iller arasında dağılımı

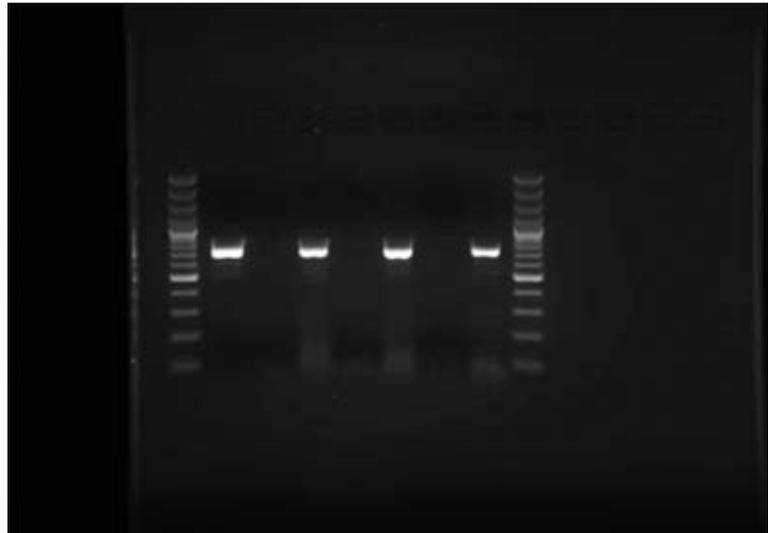
| Provinces | Cattle | Sheep | Goat |
|-----------|--------|-------|------|
| Ankara | 12 | 22 | 2 |
| Yozgat | - | 2 | 1 |
| Eskişehir | 1 | 4 | - |
| Çankırı | 68 | 5 | - |
| Çorum | 3 | 2 | 1 |
| Bartın | 3 | - | - |
| Bolu | 9 | 3 | 1 |
| Kayseri | - | 1 | - |
| Kastamonu | 3 | 1 | - |
| Kırşehir | 3 | 3 | - |
| Kırıkkale | - | 1 | - |
| Nevşehir | - | 1 | - |
| Total | 102 | 45 | 5 |

Fig 1. The figure of examined samples for *C. burnetii* by Trans-PCR (Left to right)

Lane1: Marker; 100 bp DNA ladder, Lane 2: DNA extracted from positive strain containing the gene coding phase II antigen (positive control), Lane 3: Negative control (deionized water), Lane 4-6-8: *C. burnetii* positive abomasal contents, Lane 5-7: *C. burnetii* negative abomasal contents, Lane 9: Marker; 100 bp DNA ladder

Şekil 1. Trans-PCR ile *C. burnetii* yönünden incelenen örnekler (Soldan sağa)

Kuyucuk 1: Marker; 100 bp DNA merdiveni, Kuyucuk 2: Faz II antijenini kodlayan geni içeren pozitif suştan ekstrakte edilen DNA (pozitif kontrol), Kuyucuk 3: Negatif kontrol (deionize su), Kuyucuk 4-6-8: *C. burnetii* pozitif mide içeriği, Kuyucuk 5-7: *C. burnetii* negatif mide içeriği, Kuyucuk 9: Marker; 100 bp DNA merdiven



water and 3 µl template. Cycling parameters were as follows: initial denaturation at 95°C for 2 min followed by 5 cycles of denaturation at 94°C for 30 sec, 66 to 61°C (the temperature was decreased by 1°C between consecutive steps) for 1 min, extension 72°C for 1 min and final extension 72°C for 10 min. Trans-PCR was performed using Thermal Cycler (Arktik, Thermoscientific, Germany).

Agarose Gel Electrophoresis

PCR products were electrophoresed on a 1.5% agarose gel in TBE buffer (Thermo Scientific, Vilnius, Lithuania) containing 0.5 µ/ml of ethidium bromide at 100 V for 45 min and visualized under UV light.

RESULTS

In this study, 152 abomasal contents of the aborted fetuses were sent to Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, from the provinces of Central Anatolia, and Black Sea Region of Turkey. Out of 152 abomasal contents, 11 (7.23%) were determined as *C. burnetii* positive. Four (3.92%) out of 102 cattle, 5 (11.11 %) out of 45 sheep, and 2 (40%) out of 5 goat abomasal contents were found to harbor *C. burnetii* DNA (Table 2). Of the determined *C. burnetii* positive samples, 2 positivity each belonging to 1 cattle and 1 goat were detected in the samples sent from the provinces of Black Sea Region, Bartın and Bolu, respectively (Table 3). Nine out of 11 positivity were detected in abomasal contents of the aborted fetuses delivered to the laboratory from provinces of Central Anatolia Region. Four (1 sheep and 3 cattle)

out of 11 *C. burnetii* positive abomasal contents were from Çankırı. Two out of 11 positivity were determined in the abomasal contents of aborted sheep fetuses from Ankara. The other 2 sheep abomasal contents harboring *C. burnetii* DNA were from Eskişehir and Yozgat. The last positive sample belonged to a goat from Çorum (Table 3). Remaining 141 (92.76%) abomasal contents were found to be negative for *C. burnetii* (Table 2).

DISCUSSION

A few serological surveys investigating seroprevalence of Q fever in human, sheep, cattle, and goat were implemented, but PCR-based studies were limited in Turkey [17-21]. There were few PCR-based studies, conducted on detection of *C. burnetii* in animal clinical specimens such as blood and milk. Kirkan et al. [22] detected *C. burnetii* DNA in 6 of 138 cattle blood samples by PCR. Ongor et al. [23] found that, of the examined 400 sheep milk samples, 14 (3-5 %) were *C. burnetii* positive by IMS-PCR. Dogru et al. [24] could not detect any *C. burnetii* DNA in milk samples belonging to seropositive cattle and sheep. To our knowledge, till now, neither vaginal, placental, birth fluids nor abomasal contents of aborted fetuses had been investigated for the presence of *C. burnetii* by PCR in Turkey. Nevertheless, other researchers from Turkey investigated *C. burnetii* DNA in the abomasal contents of aborted ruminant fetuses in a study conducted in Cyprus [25]. To sum up, this study was the first for the detection of *C. burnetii* from abomasal contents of aborted fetuses from ruminants by Trans-PCR in Turkey.

Table 2. The results of the examined samples for *C. burnetii* by Trans-PCR

Tablo 2. Trans-PCR ile *C. burnetii* yönünden incelenen örneklerin sonuçları

| Sample Type | Number of examined Sample | Number of Positive Samples (%) | Number of Negative Samples (%) |
|-------------|---------------------------|--------------------------------|--------------------------------|
| Cattle | 102 | 4 (3.92%) | 98 (96.07%) |
| Sheep | 45 | 5 (11.11%) | 40 (88.88%) |
| Goat | 5 | 2 (40%) | 3 (60.00%) |
| Total | 152 | 11(7.23%) | 141(92.76%) |

Table 3. Distribution of *C. burnetii* positive samples among ruminants and provinces

Tablo 3. *C. burnetii* pozitif örneklerin ruminantlar ve iller arasında dağılımı

| Provinces | Cattle | Sheep | Goat |
|-----------|--------|-------|------|
| Ankara | - | 2 | - |
| Yozgat | - | 1 | - |
| Eskişehir | - | 1 | - |
| Çankırı | 3 | 1 | - |
| Çorum | - | - | 1 |
| Bartın | 1 | - | - |
| Bolu | - | - | 1 |
| Total | 4 | 5 | 2 |

Various clinical samples such as milk [26], feces [7], urine [27], abomasal contents [25,28], vaginal mucus [29], blood [22,30], serum [31] and semen [32] are used for PCR investigation of *C. burnetii* DNA. *C. burnetii* in veterinary matrices are found in birth materials, like amniotic fluids and placentas [33,34], and in lower quantities in milk [35] and blood [9].

Before sampling, the stage of the infection and the sample type has to be well decided. In abortion, infection in placenta could spread to the fetus by the amniotic-oral route. This occurs after the penetration of placenta when the bacteria contaminate the amniotic fluid and become aspirated/swallowed by the fetus. Haematogenous spread through the umbilical vessels could be another cause of bacterial presence in fetuses and the following abortion [36].

Therefore, in this study, abomasal contents of 152 aborted fetuses comprising sheep, cattle, and goat were preferred as the material. And also, the superiority of the abomasal contents is due to comprising all of the vaginal, placental and even birth fluids [28]. Solely abomasal contents rather than other clinical samples (vaginal swabs, placental tissues, fetal lung, fetal liver, etc.) were sampled and PCR investigated in this study, therefore we cannot comment on possible presence of the agent in other tissues or samples. Similar to our study, in a study conducted in Cyprus, the researchers collected both abomasal contents and placental cotyledons from the aborted ruminants representing cattle, goat, sheep and performed trans-PCR and CB-PCR [25]. The positivity of 32.2% from placental cotyledons and 37% from abomasal contents were found by both trans-PCR and CB-PCR. The difference was attributed to the contamination of placental tissues (cotyledons) with fecal material during abortion and the positive results by both trans-PCR and CB-PCR from abomasal contents were accepted for the final decision. Parallel to our study, Dehkordi et al. [28], preferred the abomasal contents from the aborted fetuses in order to diagnose Q fever since, those were not depend on the sampling time and also as aforementioned, abomasal contents was declared harboring all of the vaginal, placental and even birth fluids. The same authors found 98 (12.53%) and 122 (16.39%) out of 782 and 744 ovine and caprine aborted fetuses were positive for the presence of *C. burnetii* by nested PCR with com1 primers, respectively. They analyzed the same samples by real-time PCR and showed that real-time PCR with trans-F and trans-R primers was 1.5 times sensitive than nested PCR [28].

The epidemiology of Q fever in Turkey is essentially unknown and to the authors' knowledge, the prevalence rate of *C. burnetii* in ruminant's aborted fetuses in Turkey has not been reported yet. Although our study was not a prevalence study, we compared Q fever positive results from the abomasal contents of aborted fetuses detected by trans-PCR with the results of similar studies from the aborted fetuses. The prevalence rate of *C. burnetii* in aborted sheep in this study in Turkey (8.88%) was found to be lower than Iran (15.47%) [28], Italy (10%) [34], Netherlands (80%) [37], Cyprus (33%) [25] and approximately the same with northern Spain (9%) [38]. While the prevalence rate of *C. burnetii* in aborted goat fetuses in this study (40%) was higher than Iran (20.43%) [28], Italy (21.5%) [39], United Kingdom (25%) [40], it was found to be lower than Netherlands (up to 80%) [37], Cyprus (50%) [25]. *C. burnetii* in aborted cattle fetuses in this study (3.92%) was found to be lower than Italy (11.6%) [39] and Cyprus (35%) [25].

In our study, 2 out of 11 positivity were determined from Bartın (1 cattle) and Bolu (1 goat), the provinces of Black Sea Region who has a Q fever history among the human. In 2002, in the Black Sea Region of Turkey, Gozalan et al. [19], declared the human Q fever outbreak and

attributed the outbreak to sheep and goat parturitions. Nine out of 11 positivities were detected from provinces of Central Anatolia Region. Although Q fever is commonly seen in the mountainous regions whose climate is tropical, Bartın and Bolu, located in west Black Sea Region have a similar climatic features such as rainy-moderate in the coastal area, dry-continental inward of the mountains and geographical features such as semi-mountainous. The climatic and geographic feature of Central Anatolia Region is continental and flat, respectively. Therefore, 11 positivity were attributed to resistance of *C. burnetii* spores to environmental conditions such as dry, harsh weather and dry atmosphere might enhance the dispersion of aerosols in those regions [41].

As a conclusion, to our knowledge this is the first study on molecular investigation of *C. burnetii* presence in stomach contents of aborted fetuses of ruminants in Turkey to date. We recommend fresh and aseptically sampled fetal stomach contents as the most appropriate materials to investigate either presence or prevalence of abortifacient pathogens, since other materials like placenta, amniotic fluid, etc. are prone to contamination by both genital flora bacteria and environmental bacteria which can lead to misidentification of causative bacteria. As previously stated by Sargison [42], amniotic fluid and fetal stomach content of healthy ewes and fetuses are sterile, so identification of bacteria in smears or culture of stomach contents of recently aborted fetuses indicates placental infection. Thus, PCR detection of *C. burnetii* DNA in fetal abomasum contents is a significant finding of a possible *C. burnetii* related abortion.

For the further studies, the relationship among the *C. burnetii* isolates detected in different geographical regions and/or the necessity of the epidemiological studies depending on the circulation of *C. burnetii* isolates within different ruminants in different and/or the same geographic regions should be taken into consideration.

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