



Detection of *C. burnetii* in Uterine Samples Collected from Referred Dogs to the Veterinary Hospital of Shahid Bahonar University of Kerman by Nested Trans-PCR

Kerman Shahid Bahonar Üniversitesi Veteriner Hastanesi'ne Getirilen Köpeklerden Toplanan Uterus Örneklerinde Nested Trans-PCR ile *C. burnetii* Saptanması

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Abstract

Q fever, a universal zoonotic disease, is caused by *Coxiella burnetii*. Dogs are considered as a reservoir of this bacterium and can infect people and other animals by shedding this pathogen in excretions. In this study, we determine the existence of the causative agent of Q fever in the uterine samples of dogs attending to the veterinary animal hospital of Shahid Bahonar University of Kerman. Specimens were collected and assessed for the presence of *C. burnetii* by nested Trans-PCR. *C. burnetii* was molecularly identified in 5 out of 41 (12.1%) col-

lected samples. This study is the first known identification of *C. burnetii* in the uterine of dogs in Kerman, Iran. According to our findings, dogs should be considered as an important source of this zoonotic pathogen. Detection of *C. burnetii* in uterine samples emphasizes the need for the further investigations on Q fever in small animals in this area.

Keywords: Q fever, *Coxiella burnetii*, uterine samples, nested trans-PCR, dog, Iran

Öz

Tüm dünyada yaygın zoonotik bir hastalık olan Q-Humması'nın etkeni *Coxiella burnetii*'dir. Köpekler bu bakterinin rezervuarıdır ve tüm ekskretleri ile etkeni saçarak insanları ve diğer hayvanları enfekte edebilirler. Bu çalışmada Kerman Shahid Bahonar Üniversitesi Veteriner Hastanesi'ne getirilen köpeklerin uterus örneklerinde Q-Humması etkeninin varlığını belirledik. Örnekler toplanarak Nested Trans-PCR ile *C. burnetii* varlığı değerlendirildi. Toplanan 41 örneğin 5'inde (%12,1) etken moleküler düzeyde tanımlendi. Bu çalışma, İran Ker-

man'da köpeklerin uterus dokularında saptanmış ilk *C. burnetii* olgusudur. Bulgularımıza göre köpekler, bu zoonoz hastalığın önemli bir kaynağıdır. Uterus örneklerinde etkenin saptanması, bu bölgedeki küçük hayvanlarda Q-Humması'nın daha ayrıntılı bir şekilde araştırılması gerekliliğini vurgulamaktadır.

Anahtar kelimeler: Q Humması, *Coxiella burnetii*, uterus örnekleri, nested trans-PCR, köpek, İran

Introduction

Query fever (Q fever) is a common and important zoonosis with worldwide distribution. It is caused by *Coxiella burnetii*, an obligate, intracellular, gram-negative bacteria (Greene, 2012). A wide range of animals encompassing wild and domestic mammals, birds and arthropods are considered as Q fever res-

ervoirs (Gwida et al., 2012; Agerholm, 2013). In animals, the *C. burnetii* infection is mostly subclinical and infected cases are rarely symptomatic (Greene, 2012; Kopency et al., 2013). However, reproductive disorders including abortions, stillbirths, retained placenta, infertility and weak newborns have been determined in affected animals (Cantas et al., 2011; Gwida et al., 2012). Dogs can be infected via contact with infected live-

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stock and exposure to by-products as well as through tick vectors (Havas and Burkman, 2011). A large number of bacteria is excreted in milk, feces, urine, vaginal mucus, and, particularly, birth products by infected animals (Kilic et al., 2008; Porter et al., 2011). Ingestion and inhalation of environmentally resistant organisms are two main routes of transmission in animals and humans (Greene, 2012; Knobel et al., 2013). In humans, Q fever manifests sub-clinically with no clinical signs to either chronic or acute disease which may result in life-threatening conditions and even death (Skerget et al., 2003; Cooper et al., 2011; Norris et al., 2013). DNA-based methods have been successfully used for the detection of *C. burnetii* in tissue samples (Greene, 2012). This re-emerging disease poses a public health concern, and therefore, it has been gaining increasingly more attention from physicians and veterinarians in recent years. Asymptomatic infected animals, particularly in times of parturition, can be a source of infection for people who are in contact with these animals (Greene, 2012). Even though Q fever is endemic in Iran and most human cases have been related to livestock exposure (Ezatkah et al., 2014), limited data about the distribution of the disease in companion animals is available. The purpose of this study was to detect the presence of *C. burnetii* DNA in uterine tissues of dogs in Kerman, southeast Iran using nested Trans-PCR assay.

Materials and Methods

Sample collection and DNA extraction

The target group was comprised of 41 dogs including 26 shelter and 15 client-owned female dogs presented to the Veterinary Teaching Hospital of Shahid Bahonar University of Kerman regardless of their age and clinical status for ovariohysterectomy from April 2014 to October 2016. A full-thickness uterine sample (a 3×3 mm piece) was taken at ovariohysterectomy from each dog. The collected samples were separately transferred into sterile Eppendorf tubes and preserved in 70% ethanol. *C. burnetii* was extracted from uterine samples using the DNA extraction Mini Kit (Yekta Tajhiz Azma, Iran), according to the manufacturer's instructions. The extracted DNA was preserved at -20°C upon arrival.

Standard strain of *C. burnetii*

The DNA from the *C. burnetii* standard Nine Mile, phase II, strain (RSA 493) and sterile distilled water were used as the positive and negative controls respectively.

Molecular detection of bacterium (nested Trans-PCR assay)

In this study, nested Trans-PCR assay was carried out for the detection of *C. burnetii* in the uterine samples. The primers Trans 1- Trans 2 and 261 F- 463 R were used and designed based on a repetitive, transposon-like element (Trans-PCR) as previously described (Berri et al., 2000; Parisi et al. 2006). A positive control with 6 ng of *C. burnetii* DNA as the template and a negative control without a DNA template were considered in each PCR run. The sequences of the primers used in this study are displayed in Table 1.

Nested Trans-PCR amplifications were done via two runs of PCR using two sets of primers including Trans1 and Trans2 for the first amplification followed by 261F and 463R for the second amplification reaction. The volume was 25 µL containing 5 µL of DNA template, 12.5 µL/reaction commercial master mix (Hot Start Qiagen Master Mix), 4.5 µL water, 1.5 µL of each primer at a concentration of 10 mM. DNA amplifications were performed in a MG thermal cycler (Eppendorf, Germany). The first amplification of PCR was 95 °C for 2 min, followed by five cycles at 94°C for 30 s, 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, then a final extension step of 10 mins at 72°C.

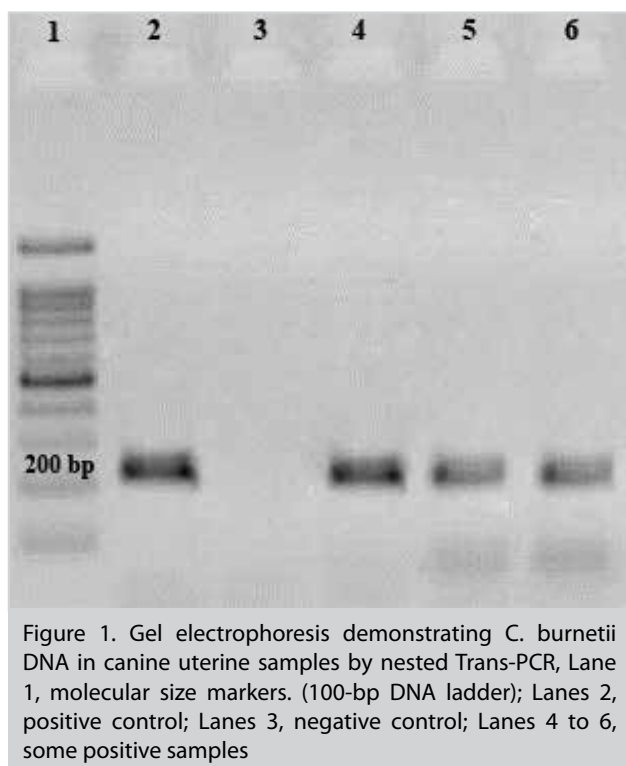
Then, 5 µL of the first amplification product was subjected to the second amplification with the nested primers. In the second amplification, the cycling conditions included an initial denaturation of DNA at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 50°C for 45 s, 72°C for 1 min, then a final extension step of 10 mins at 72 °C. Amplicons were analyzed by the agarose gel electrophoresis, stained with fluoro dye, visualized under UV light, and finally, photographed.

Results

In the present study, uterine samples from 15 pet and 26 stray dogs (41 in total) were acquired and assessed for the presence of *C. burnetii* using nested Trans-PCR. *C. burnetii* DNA was detected from five of the 41 (12.1%) uterine biopsies of dogs. No signal was detected in the negative control (Figure 1). Two of the 15 uterine samples of pets were positive for *C. burnetii* while in the stray group, 3 positive cases were detected. Of these five positive samples, one pet had pyometra and other pet had post-partum metritis. All three shelter dogs which tested positively were apparently healthy with unknown history.

Table 1. Characteristics and sequences of nested Trans-PCR primers for *C. burnetii* detection

Protocol	Primer	Sequence	Gen	Amplicon	Reference
Trans-PCR	Trans1	5-TATGTATCCACCGTAGCCAGT C-3	IS1111	687	Berri et al. 2000
	Trans2	5-CCCAACAACACCTCCTTATTC-3			
Nested PCR	261F	5-GAGCGAACCATTTGGTATCG-3	IS1111	203	Parisi et al. 2006
	463R	5-CTTTAACACGCGCTTGAACGT-3			



Discussion

Pets, particularly cats, are known to be a significant reservoir of Q fever outbreak in the Urban setting (Greene, 2012; Hornok et al., 2013). Following a Q fever outbreak in a Sydney veterinary hospital, the role of companion animals in *C. burnetii* transmission to humans was emphasized (Maywood and Boyd, 2011). Based on previous reports, most human cases had a history of exposure to breeding queens or bitches and neonates during parturition (Marrie et al., 1988 a,b; Marrie et al. 1989; Norris et al., 2013). In this study, we assess the presence of *C. burnetii* DNA in the uterine tissues of dogs. Our data show that the uterine of dogs can be infected by *C. burnetii*.

In literature, the varying prevalence of *C. burnetii*, ranging from 0 to 35% was serologically reported in dogs throughout the world (Havas and Burkman, 2011; Cooper et al., 2011; Norris et al., 2013). Furthermore, the seroprevalence of *C. burnetii* infection in cats varies from 1.5% to 42% in different areas (Matthewman et al., 1997; Kilic et al., 2008; Kopečný et al., 2013; Norris et al., 2013). Q fever seroprevalence was also found to be 6.2% to 32% in maritime Canada and contact with parturient cats and newborn kittens has been identified as an important risk factor for humans acquiring Q fever (Marrie et al., 1988 a,b; Marrie et al., 1989; Norris et al., 2013). Moreover, Kopečný et al. (2013) serologically found *C. burnetii* in breeding cattery-confined cats. Two out of 27 (7.4%) cats were positive using CFT and 7 out of 27 (26%) and 6 out of 27 (22%) cats were positive for anti-phase II and I *C. burnetii* antibodies using IFA respectively. Eleven out

of 27 (41%) cats were seropositive using ELISA. According to the study by Komiya et al. (2003), cats are considered as the most important reservoirs of *C. burnetii* in Japan. In the study, the prevalence of *Coxiella* infection was serologically higher in stray cats compared with pet cats.

We detected *C. burnetii* in the DNA of five of the 41 (12.1%) uterine biopsies of dogs in the current study. In comparison, this bacterium has been isolated from the reproductive organs of healthy and ill dogs and cats (Greene, 2012). Fujishiro et al. (2015) evaluated the presence of *C. burnetii* DNA in uterine biopsies of cats with or without clinical and histopathological evidence of reproductive problems. In this study, *C. burnetii* DNA was detected in three of 37 samples using the IS1111 PCR. In another study performed in Colorado, *C. burnetii* was detected in 8.5% of uterine samples of clinically healthy, non-parturient client-owned cats. Sequencing of nucleotide in the study showed 99% homology to *C. burnetii* DNA (Cairns et al., 2007). This author indicated "that apparently healthy cats could serve as a source of human *C. burnetii* infection". This is in contrast to a study in the Netherlands in which *C. burnetii* DNA was not found in any placentas of breeding cats following the 2007–2010 Q fever outbreak (Roest et al. 2013); However, *C. burnetii* was found in 4 (7%) out of 54 canine placentas in this study.

Q fever in animals is usually asymptomatic. Apparently healthy infected animals shed organisms in excretions especially in birth by-products (placenta) and contaminate the environment (Greene, 2012). Reproductive failures including placentitis, abortion, stillbirths, prematurity, low birth weight, neonatal weakness, death of kittens in females and orchitis, epididymitis and priapism in males can be linked to the *C. burnetii* infection (Agerholm, 2013; Kopečný et al., 2013). Chronically infected animals mostly colonize *C. burnetii* in the uterus and mammary glands and shed over 10^9 bacteria into the environment during parturition (Gwida et al., 2012). Exposure to parturient dogs and cats delivering stillborn or healthy kittens has resulted in several outbreaks of human Q fever (Agerholm, 2013). In dogs, any evidence of reproductive disorders associated with this pathogen have not been established and clinical aspects remain obscure (Agerholm, 2013). However, a human case of Q fever was reported related to contact with an infected parturient dog whose four pups died after birth (Buhariwalla et al., 1996). In cats, bacterium was detected from genital tracts of healthy cats and animals with reproductive disorders (Agerholm, 2013; Fujishiro et al., 2015). In this study, two out of 15 uterine samples of pets were positive for *C. burnetii* while in the stray group, 3 positive cases were detected. Of these five positive samples, one pet had pyometra and the other pet had post-partum metritis. All three positive shelter dogs were apparently healthy with unknown history. The small sample size and majority of stray dogs are a problem of this study precluding statistical comparison between groups. A positive relationship was also demonstrated between contact with wildlife and farm animals, and tick exposure (Cooper et al., 2011). Stray

dogs and catsexposed to products and body fluids/parts of farm animals show a high prevalence (Greene, 2012; Hornok et al., 2013). In contrast, Komiya et al. (2003) found *C. burnetii* DNA in uterine samples of client-owned cats.

Pet ownership, especially dogs and cats, is considered a significant risk factor for the transmission of zoonotic diseases. As interest in pet ownership in Iran actively continues to grow, the importance of this zoonotic disease is increasing. Infected animals, especially asymptomatic ones, are a potential source of infection for humans. According to the previous study, Q fever is endemic to Iran (Mostafavi et al., 2012). Ruminants as well as ticks were documented to carry *C. burnetii* (Khalili and Sakhaee, 2009; Khalili et al., 2010; Sakhaee and Khalili, 2010; Khalili et al., 2012; Asadi et al., 2014; Ezatkah et al., 2014; Khalili et al., 2015), followed by tevidence of human infections in the southeast of Iran (Khalili et al., 2010; Ezatkah et al., 2014; Khalili et al., 2014). Also, drought and low rainfall predispose the transmission of aerosols of *C. burnetii* in this area (Ezatkah et al., 2014).

This study determines that the uterus of dogs in southeast Iran can harbor *C. burnetii*. Contact with aborting or parturient dogs, parturient secretions and new kittens should be undertaken with care. According to the results of the present study and other reports (Cairns et al., 2007), early ovariohysterectomy of companion animals may be recommended, particularly for pets of immune suppressed individuals. Our findings indicate that *C. burnetii* is a significant pathogen of concern in Iran. *C. burnetii* and can be extremely hazardous to humans in view of its high transmissibility and low infectious dose (Gwida et al., 2012). Therefore, the early diagnosis and treatment of infected dogs, sanitary and prophylactic measures and ectoparasite control should be done to prevent outbreaks Q fever (Skerget et al., 2003; Havas and Burkman, 2011; Porter et al., 2011). Better understanding of the pathogenesis of this bacterium in companion animals leads to better prevention and control of Q fever outbreaks. The role of *C. burnetii* in reproductive abnormalities in dogs and cats should be evaluated.

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