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Article

Detection of *Coxiella burnetii* (Gammaproteobacteria: Coxiellaceae) in ticks collected from infested dogs in Kerman, Southeast of Iran

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ABSTRACT
Q fever, as one of the tick-borne zoonotic diseases, is caused by *Coxiella burnetii*. Ticks may play an important role in *C. burnetii* transmission to animals and humans. By using nested Trans-PCR, we investigated the presence of *C. burnetii* in ticks collected from dogs in Kerman, southeast of Iran. A total of 375 ticks were randomly collected from 100 dogs. Eight pools were finally formed. The pools of tick samples were assessed for the presence of *C. burnetii*. Genomic DNA extraction was done and samples were evaluated by nested Trans-PCR. All tick specimens were identified as *Rhipicephalus sanguineus* regarding the taxonomical characteristics. *Coxiella burnetii* was detected in 1 out of 8 (12.5%) pool samples. One positive sample was subjected to sequence analysis, which successfully confirmed the accuracy of the PCR assay. Our data show that ticks infesting dogs can be infected by *C. burnetii*, providing zoonotic importance of these populations. Efforts should be focused on understanding the role and epidemiologic importance of dogs and their ticks, especially for human Q fever, which can be a life-threatening disease.

KEY WORDS: Genomic DNA extraction; nested Trans-PCR; Q fever; *Rhipicephalus sanguineus*; zoonotic disease.

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INTRODUCTION

Tick-borne diseases are among the most prevalent problems diagnosed in both the medical and veterinary medicine and their spectrum has been recently increased (Dantas-Torres *et al.* 2012). Ticks are obligate hematophagous arthropods which can be transported over large distances by animals and serve as vectors of pathogens (Dantas-Torres *et al.* 2012; Socolovschi *et al.* 2012). Q fever, as one of the tick-borne zoonotic diseases, is caused by *Coxiella burnetii* (Cooper *et al.* 2011; Greene 2012; Norris *et al.* 2013). Q fever is universally considered as a public health concern (Porter *et al.* 2011; Kopecny *et al.* 2013). Two major modes of transmission of this bacterial disease are inhalation and ingestion of the organisms (Porter *et al.* 2011; Greene 2012). A wide variety of wild and domestic mammals, birds and their respective ectoparasites have been mentioned as *C. burnetii* reservoirs (Mediannikov *et al.* 2010; Greene 2012; Kopecny *et al.* 2013). It is demonstrated that more than 40 species of ticks can be infected with *C. burnetii*. So, they may play an important role in *C. burnetii*

transmission to animals and humans by excretion of this pathogen in feces, saliva and coxal fluid (Sprong *et al.* 2012; Knobel *et al.* 2013). Following tick bites, dogs can also acquire the *C. burnetii* infection (Havas and Burkman 2011; Greene 2012). Q fever often manifests as an asymptomatic, self-limited illness in humans and animals which can be either acute or chronic, ranging from no clinical signs to sudden death (Cooper *et al.* 2011; Greene 2012; Norris *et al.* 2013). Diagnosis of Q fever is based on serologic testing or organism isolation (Greene 2012; Kopecny *et al.* 2013). *Polymerase Chain Reaction* (PCR) especially Trans-PCR offers other laboratory methods to detect *C. burnetii* in various samples such as ticks (Vaidya *et al.* 2008; Porter *et al.* 2011).

Q fever is an endemic widespread zoonosis (Greene 2012). Pet ownership continues to grow in Iran that besides many advantages poses health concern regarding acquirement of zoonotic pathogens. Dogs and their ticks can transmit *C. burnetii* infection to humans, and this issue increases the number of Q fever outbreaks (Greene 2012; Norris *et al.* 2013). Despite the considerable importance of this challenging disease, studies regarding *C. burnetii* infection in dogs and ticks infesting them are scant. To the knowledge of the authors, there are no reports about it in Iran. We investigated whether ticks feeding on dogs may be involved in *C. burnetii* transmission. So, the presence of *C. burnetii* DNA in ticks collected from dogs in Kerman, southeast of Iran, was evaluated by using nested Trans-PCR.

MATERIAL AND METHODS

Tick collection and DNA extraction

A total of 100 dogs that were referred to the Veterinary Teaching Hospital of Shahid Bahonar University of Kerman from April 2012 to October 2013, were considered as the target population, regardless of their age, sex and clinical status. After general examination, each dog was inspected for the presence of ectoparasites. Then, a maximum of ten attached, semi- or fully engorged ticks were manually collected from infested dogs using forceps. The specimens were separately transferred into the labeled holding tubes containing absolute ethanol and sent to parasitology laboratory for identification. In the laboratory, collected ticks were identified by using a binocular microscope (40x magnification). Ticks specifically were identified according to Rahbari *et al*. (2008).

DNA was extracted from tick samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was preserved at –20 °C until analyses. From a total number of 375 ticks, 8 pools were finally formed. The DNA from the *C. burnetii* standard Nine Mile, phase II, strain (RSA 493, Slovakia), and sterile water was used as the positive and negative control, respectively.

PCR assay and Sequencing

Nested PCR assay was done for detection of *C. burnetii* genome within the tick's body according to the methods of Berri *et al.* (2000) and Parisi *et al.* (2006). The primers used in the current study are shown in Table 1.

Table 1. The nested Trans-PCR primers for nested PCR *icd* gen.

Target	Primer	Sequences 5'-3'	Amplicon (bp)
icd	icd1-F	CGGAGTCTCTTAGTGATGACGGA	400
	icd5-R	GCAGCGCGACATTGAGCGAACG	
	icd1N-F	GGAGTTAACCGGAGTATCCA	370
	icd2N-R	ATTGAGCGAACGTATGCCAC	

The first amplification of the nested PCR was done in a total volume of 25 µl containing 5 µl of DNA sample, 2 U of *Taq* DNA polymerase (Takapouzist Co., Iran), and final concentrations of 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 3 mM $MgCl_2$; 1 mM dNTP mix and the primers at a concentration of 10 mM each. Then, 5 µl of the first amplification product was subjected to the second amplification with the nested primers. A positive control of 6 ng *C. burnetii* DNA as the template and a negative control without DNA template was considered in each PCR round. Amplification of DNA was done in the MG thermal cycler (Eppendorf, Germany)

Both amplifications were done at 95 ℃ for 3 min., followed by 35 cycles at 94 ℃ for 60 sec, 55 ℃ for 45 s, and 72 ℃ for 45 s, followed by a final extension step of 10 min at 72 ℃ (Nguyen *et al*. 1999). Amplicons were analyzed by 1.2% agarose gel electrophoresis, stained with ethidium bromide, visualized under UV light.

The PCR products were submitted to Gen fannavaran Company (Iran) for sequencing. Sequence analysis was performed on one *C. burnetii* isolate to compare with the National Center for Biotechnology Information GenBank database entries, then blasted. Thirty-four *C. burnetii* insertion sequence IS transposase gene, were obtained from GenBank. These sequences were aligned using MEGA 7.0 software. The phylogenetic tree was reconstructed by maximum likelihood phylogenetic method, using the best-fitting T92 model of nucleotide substitution.

RESULTS

In this study, a total of 375 ticks were randomly collected from 100 dogs. All the specimens were identified as *Rhipicephalus sanguineus* regarding the standard characteristics including red-brown color, elongated body shape, and hexagonal basis capituli. Eight pools were finally formed from a total number of 375 ticks. The pools of tick samples were assessed for the presence of *C. burnetii*. The bacterium was detected in one out of 8 (12.5%) pool samples (Fig. 1). The positive sample was subjected to sequence analysis best matched with *C. burnetii* from GenBank database, which successfully sequenced and confirmed the accuracy of the nested Trans-PCR assay (Accession number of GenBank: KP878696). The relationship between the genotype found in the present study and the internationally known genotypes are presented in the phylogenetic trees in Figure 2.

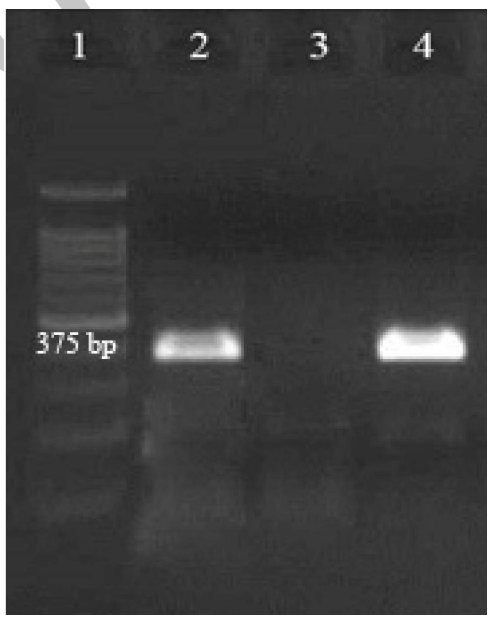

Figure 1. Detection of *C. burnetii* DNA in ticks collected from dogs by nested PCR (*icd* gen), Column 1, molecular size markers (100-bp DNA ladder); Column 2, positive control; Column 3, negative control; Column 4 positive sample.

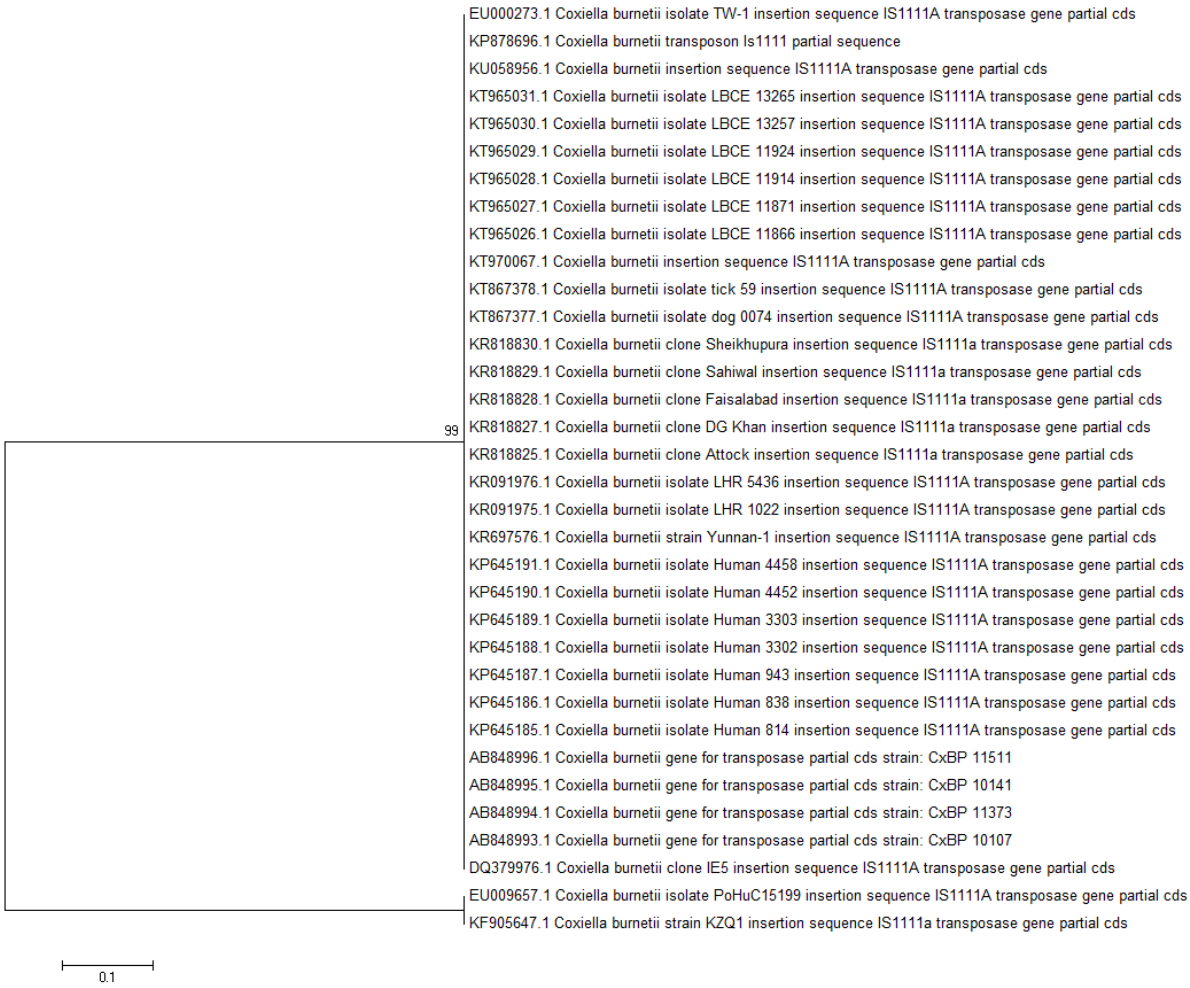

Figure 2. Phylogenetic tree with genotypes of *C. burnetii.*

DISCUSSION

The significance of tick-borne diseases is well established as their incidences are increasing worldwide. Thus, these diseases have gained more attention in the medical and veterinary medicine in recent years (Hornok *et al.* 2013). Ticks as one of the most important vectors of pathogenic agents are documented as *C. burnetii* reservoirs (Dantas-Torres *et al.* 2012; Socolovschi *et al.* 2012). This study provides the first detection of *C. burnetii*, the causative agent of Q fever, in ticks collected from dogs in Iran, using nested Trans-PCR assay

It has been well documented that more than 40 species of ticks can be infected by *C. burnetii* (Havas and Burkman 2011; Porter *et al.* 2011; Knobel *et al.* 2013). The sylvatic cycle with reservoir hosts is maintained by these arthropods. Ticks shed bacteria in saliva and feces (Porter *et al.* 2011; Sprong *et al.* 2012). Vertical transmission of *C. burnetii* infection such as transstadial and transovarial transmission is known in various species of ticks (Toledo *et al.* 2009; Sprong *et al.* 2012). Moreover, animals and humans may be involved following a tick bite (Greene 2012). Crushing infected ticks may also cause Q fever (Mediannikov *et al.* 2010). In the present study, *C. burnetii* was detected in ticks collected from dogs, suggesting that ticks may play an important role in the *C. burnetii* transmission in this area. According to literature, guinea pigs were experimentally infected by *C. burnetii* with *Ixodes holocyclus*, *Haemaphysalis bispinosa*, *Rhipicephalus sanguineus,* and *Dermacentor andersoni* (Porter *et al.* 2011). This challenging bacterium was naturally isolated from *Ixodes ricinus* and *Rhipicephalus sanguineus* (Greene 2012). In accordance with our finding,

Socolovschi *et al.* (2012) isolated *C. burnetii* from ticks feeding on dogs, horses, cats and humans (Socolovschi *et al.* 2012). Additionally, the high prevalence of *C. burnetii* in ticks collected from domestic animals (cattle, goats, sheep, horses, and donkeys), was observed by Mediannikov *et al.* in 2010. Also, one tick sample collected from the cat was positive in another study (Sprong *et al.* 2012).

We found *C. burnetii* in one out of 8 (12.5%) pool samples of ticks collected from dogs. All the ticks were identified as *Rhipicephalus sanguineus* in the present study. In comparison, *C. burnetii* was detected in five out of 10 pools (50%) of ticks collected from domestic dogs in Rural Western Kenya using *IS1111* qPCR in another study (Knobel *et al.* 2013). In the mentioned study, the percentage rates of the tick species were as follows: *Rhipicephalus sanguineus* (20%), *R. appendiculatus* (11.1%), unspeciated *Rhipicephalus* (20%), *Amblyomma variegatum* (20%), *Haemaphysalis leachi* (50%), and *R.* (*Boophilus*) *decoloratus* (20%) (Knobel *et al.* 2013). In another report performed in Spain, 3.4% of ticks collected from animals, including *Hyalomma lusitanicum*, *Dermacentor marginatus*, *R. sanguineus*, and *R. pusillus* were positive; however, *C. burnetii* was not detected in ticks collected from pets (Toledo *et al.* 2009). In contrast, some researchers could not determine *C. burnetii* infection in tick samples (Sprong *et al.* 2012; Astobiza *et al.* 2011). It seems that the differences between results of various studies are related to variation between evaluated populations, host factors, geographical and environmental conditions, and identification methods. Thus, comparison of various studies should not be done without attention to these issues. In this study, we used nested Trans-PCR to detect *C. burnetii* in ticks. This method has proven to be highly specific and sensitive, as it detects even very few copies of a precise DNA sequence (Parisi *et al.* 2006; Porter *et al.* 2011). We also used pools of ticks in order to facilitate the possibility of *C. burnetii* detection.

Pets have been documented as *C. burnetii* reservoir throughout the world (Greene 2012). Many researchers have determined *C. burnetii* infection in sera or tissue samples of dogs, suggesting the zoonotic importance of dog populations (Cooper *et al.* 2011; Havas and Burkman 2011; Hornok *et al.* 2013; Norris *et al.* 2013; Roest *et al.* 2013). So, ticks can be contaminated during feeding on infected dogs. In contrast, healthy dogs may be involved after being bitten by infected ticks (Greene 2012). Hence, outdoor housing (contact with the farm, wildlife, and ticks) and feeding by raw diet may be attributed to the higher prevalence of Q fever in dogs, maintaining *C. burnetii* in the environment (Cooper *et al.* 2011; Hornok *et al.* 2013).

At present, few studies have been done about the distribution and incidence of *C. burnetii* infection in Iran. In the southeast of Iran, recent studies showed a high seroprevalence of *C. burnetii* in domestic ruminants (Khalili and Sakhaee 2009; Sakhaee and Khalili 2010) in agreement with the high prevalence of human Q fever cases reported from this area (Khalili *et al.* 2010; Khalili *et al.* 2014). Nourollahi Fard and Khalili (2011) also detected *C. burnetii* in ticks collected from sheep and goats. In our region, domestic ruminants are the main *C. burnetii* reservoir and being exposed to this population and their products may lead to a higher prevalence of *C. burnetii*. Indeed, commercial or home-made diets are infrequently used for dogs, and most of them feed on a raw diet such as carcasses.

Our data showed that ticks infesting dogs can be infected by *C. burnetii*, providing zoonotic importance of these populations (dogs and their ticks). As pet ownership continues to grow in Iran, optimization of sanitary and prophylactic measures, the especially appropriate tick control strategy is essential to prevent a Q fever outbreak. Efforts should be focused on understanding the role and epidemiologic importance of dogs and their parasitized ticks, especially for human Q fever, which can be a life-threatening disease.

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ردیابی *Coxiella burnetii* (Gammaproteobacteria: Coxiellaceae) در کنه‌های جمع آوری شده از سگ‌های آلوده در کرمان، جنوب شرق ایران

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چکیده

تب کیو به عنوان یکی از بیماریهای مشترک کنه‌زاد توسط *Coxiella burnetii* ایجاد می‌شود. کنه ممکن است نقش مهمی در انتقال *Coxiella burnetii* به حیوانات و انسان‌ها داشته باشد. با روش nested Trans-PCR، وجود *Coxiella burnetii* در کنه‌های جمع آوری شده از سگ‌ها در کرمان، جنوب شرق ایران مورد بررسی قرار گرفت. در مجموع ۳۷۵ کنه به شکل تصادفی از ۱۰۰ سگ جمع آوری شد. هشت مخزن در نهایت شکل گرفت. مخازن نمونه‌های کنه برای وجود *Coxiella burnetii* ارزیابی شد. استخراج DNA انجام شد و نمونه‌ها با روش nested Trans-PCR مورد ارزیابی قرار گرفت. تمامی نمونه‌های کنه‌ها بر اساس ویژگی‌های تاکسونومیک، *Rhipicephalus sanguineus* تشخیص داده شد. *Coxiella burnetii* در یکی از هشت نمونه‌های مخزن (۱۲/۵ درصد) تشخیص داده شد. تجزیه و تحلیل سکانس یکی از نمونه‌های مثبت درستی روش PCR را تأیید کرد. داده‌های این پژوهش نشان داد که کنهٔ سگ‌های آلوده می‌تواند با *Coxiella burnetii* آلوده شود که نشان‌دهندهٔ اهمیت زئونوتیک این جمعیتهاست. تلاش بایستی بر فهم نقش و اهمیت اپیدمیولوژیک سگ‌ها و کنه‌های‌شان متمرکز شود، به ویژه در رابطه با تب کیوی انسانی که می‌تواند بیماری تهدید کنندهٔ زندگی باشد.

واژگان کلیدی: استخراج DNA ژنومی؛ nested Trans-PCR؛ تب کیو؛ *Rhipicephalus sanguineus* ؛ بیماری زئونوتیک.

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