

Research article

MOLECULAR EVIDENCE OF Q FEVER AGENT *COXIELLA BURNETII* IN IXODID TICKS COLLECTED FROM STRAY DOGS IN BELGRADE (SERBIA)

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Q fever is a zoonotic disease caused by *Coxiella burnetii*, a gram-negative coccobacillus, which has been detected in a wide range of animal species, mostly domestic ruminants, but also in wild mammals, pets, birds, reptiles, arthropods (especially ticks), as well as in humans. Although the exposure to domestic animals in rural areas is regarded as the most common cause of the disease in humans, recent studies have shown that the role of pets in the epidemiology of Q fever has been increasingly growing. Although the primary route of infection is inhalation, it is presumed that among animals the infection circulates through ticks and that they are responsible for heterospecific transmission, as well as spatial dispersion among vertebrates. The aim of this study was to determine the presence and prevalence of *C. burnetii* in ticks removed from stray dogs, as well as to examine the distribution of tick species parasitizing dogs on the territory of Belgrade city. A PCR protocol targeting IS1111 repetitive transposon-like region of *C. burnetii* was used for the detection of *C. burnetii* DNA in ticks and the results were confirmed by sequence analysis. In total, 316 ticks were collected from 51 stray dogs - 40 females (78.43%) and 11 males (21.57%). Three species of ticks were identified: *Rhipicephalus sanguineus* (72.15%), *Ixodes ricinus* (27.53%) and *Dermacentor reticulatus* (0.32%). Out of 316 examined ticks, *C. burnetii* DNA was detected only in the brown dog tick *R. sanguineus*, with a total prevalence of 10.53% (24/228). The high prevalence of *C. burnetii* in *R. sanguineus*, which is primarily a dog tick, indicates the importance of dogs in the epidemiology of Q fever in the territory of Belgrade.

Key words: Belgrade, *Coxiella burnetii*, dogs, ticks, PCR

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INTRODUCTION

Q fever is a zoonotic disease distributed worldwide, with the exception of Antarctica and New Zealand, caused by *Coxiella burnetii*. *C. burnetii*, a gram-negative coccobacillus, has been detected in a wide range of animal species, mostly domestic ruminants, but also in wild mammals, pets, birds, reptiles, arthropods (especially ticks), as well as in humans [1]. In nature, the bacterium occurs in two morphological forms – as a large cell variant (LCV) which is metabolically active intracellular form, and the small cell variant (SCV), which is an extracellular and metabolically inactive infectious form. SCV appears as a spore-like form, and it is extremely resistant to physical and chemical factors and can survive outside the host for prolonged periods of time [2,3]. Due to its exceptional resistance and stability in the external environment, as well as the possibility of aerosol dissemination, the Centers for Disease Control and Prevention have categorized *C. burnetii* as a category B Bioagent.

The most frequently reported source of human infection is the exposure to aerosols or animal products contaminated with infected domestic ruminants' excreta. Animals excrete *C. burnetii* in feces and urine, while gravid animals shed the agent through the placenta and birth products, which represent a significant source of environmental contamination [4-7]. The main route of human infection is inhalation of dust contaminated with excreta of infected animals [8]. Ingestion of raw milk as a source of human infection still remains a subject of controversy [6]. Although the exposure to domestic animals in rural areas is regarded as the most common cause of the disease in humans, recent studies have shown that the role of pets in the epidemiology of Q fever has been increasingly raised [9-11]. Dogs can be infected by means of ingestion of infected material (birth products), inhalation [5], but also through tick bites [12]. The disease in dogs is usually asymptomatic, but infection in pregnant bitches can lead to early death of pups [9].

More than 40 tick species can carry *C. burnetii* [1] and the agent can be found in the intestine, haemolymph, Malpighian vessels, salivary glands and ovaries of ticks. It can be transmitted transovarially and transstadially [1,13]. Considering the extremely high resistance of *C. burnetii* in the external environment, it is important to note that ticks can excrete large amounts of *C. burnetii* in feces - as high as 10^{10} bact./g feces [14]. Although the primary route of infection is inhalation, it is presumed that among animals the infection circulates through ticks and that they are responsible for heterospecific transmission, as well as spatial dispersion among vertebrates [15,16].

The aim of this study was to determine the presence and prevalence of *C. burnetii* in ticks removed from stray dogs, as well as to examine the distribution of tick species parasitizing dogs on the territory of Belgrade city. This population of dogs is important for examination because these animals are completely unprotected with ectoantiparasitics, they do not receive any chemoprophylaxis, and they move freely, which increases the chance of contact with potentially infected ticks. This way, stray dogs can carry the Q fever agent to new locations. By identifying the tick species

parasitizing dogs, and the prevalence of *C. burnetii*-positive ticks, it is possible to define potential risk factors and the importance of ticks in the epidemiology of Q fever in stray dogs in Belgrade.

MATERIAL AND METHODS

Ethics statement

This research was approved by *Veterinary Directorate* of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, in accordance with the Animal Welfare Law, Law on General Administrative Procedure and Law on State Administration, Republic of Serbia, by the Decision no. 323-07-00364/2017-05/3.

Sample collection

Ticks were recovered from stray dogs, during the period from April to November, at the Faculty of Veterinary Medicine, University of Belgrade (Serbia). The animals were included in the program of sterilization and castration as part of the Strategy for solving the problem of stray dogs on the territory of Belgrade (Official Gazette of the City of Belgrade, No. 37/2011). Prior to surgery, a clinical examination of each animal was performed, and no animal showed symptoms that indicated any infectious disease. During the clinical examination, the body of each animal was also examined for the presence of ticks. Ticks were carefully removed from animals, using tweezers, and placed into glass vials with 70% ethanol. Each sample was adequately labelled, according to the animal from which it was removed, and kept at the temperature of -20 °C.

Morphological identification of ticks

Morphological identification of collected ticks was performed at the Laboratory for Parasitology at the Department of Parasitology at the Faculty of Veterinary Medicine, University of Belgrade. Identification was carried out by using a stereomicroscope (Carl Zeiss, Jena), at the 100x magnification. Ticks were classified into species and developmental stage according to standard taxonomic key [17].

DNA extraction

Prior to DNA extraction, individual ticks were rinsed in decreasing concentrations of ethanol (70%, 50% and 30%). After the final wash in sterile distilled water, the ticks were left to air dry on sterile filter paper. Each tick was then placed in a separate 1.5 ml sterile plastic microtube. In the next step, sterile phosphate-buffer saline was added to each tube and the ticks were mechanically crushed and homogenized using a sterile metal rod. During the first phase, aliquots of homogenized ticks were pooled and each pool consisted of 3-10 ticks (depending on the sample size) in a final volume of 200 µl, according to species, developmental stage and the animal from which they

were removed. The DNA was extracted in a final volume of 100 µl using a commercial DNA extraction kit (GeneJET Genomic DNA Purification Kit, Fermentas, USA), according to the manufacturer's instructions. The extracted DNA was stored at -20 °C. After obtaining positive tick pools, a DNA extraction was performed on each individual tick within the positive pools, as previously described.

PCR

A PCR protocol targeting IS1111 repetitive transposon-like region of *C. burnetii* [18] was used for the detection of *C. burnetii* DNA in ticks. For the PCR amplification, primers Trans-1 (5'-TAT GTA TCC ACC GTA GCC AGT C-3') and Trans-2 (5'-CCC AAC AAC ACC TCC TTA TTC-3') were used, yielding a specific fragment of 687 bp. The PCR reaction was performed on 10 µl of each DNA sample, at a final volume of 25 µl. The final reaction mixture consisted of 2 µM of each primer, 200 µM of each dNTP, 3 mM MgCl₂, and 0.5 U of Taq DNA polymerase (Thermo Fisher Scientific, USA). DNA amplification was performed using the Mastercycler® ep Gradient S (Eppendorf, Germany) thermal cycler. The Touchdown PCR program included the following cycles: initial denaturation of DNA at 95 °C for 2 min, followed by five cycles consisting of denaturation at 94 °C for 30", annealing from 66-61 °C (the temperature was reduced by 1 °C in each subsequent cycle) for 1 min, and extension at 72 °C for 1 min. After these five cycles, there were 35 cycles consisting of denaturation at 94 °C for 30", annealing at 61 °C for 30" and extension for 1 min at 72 °C, and finale extension of 10 min at 72 °C. Visualisation of PCR products was performed by using horizontal electrophoresis in 1% agarose gel, at voltage of 100 V for 40 min. Amplicons were stained with Midori Green DNA stain (Nippon Genetics Europe, GmbH, Germany), visualized on UV transilluminator and photographed. Positive and negative controls were used in each PCR reaction. DNA extracted from the Phase I antigen of *C. burnetii* was used as a positive control and sterile distilled water was used as a negative control instead of DNA sample. In order to verify the repeatability of the results, PCR reactions were performed twice on each sample.

Sequencing

Purification of the PCR products was performed by using a commercial purification kit (QIAquick PCR Purification Kit, Qiagen, USA) according to the manufacturer's instructions. Sequencing was done in both directions using a commercial service (Macrogen, Europe). Obtained sequences were processed and analyzed by using BioEdit software, and sequence alignment was performed by using Clustal W [19]. The sequences were compared with analogue sequences deposited in the Gene Bank using BLAST.

RESULTS

In the current study, 316 ticks were collected from 51 stray dogs - 40 females (78.43%) and 11 males (21.57%). All dogs were mixed-breed, body weight ranging from 6 to

30 kg. The number of ticks per animal was 1-42. All dogs were clinically healthy and sexually mature. In total, three species of ticks were identified: *Rhipicephalus sanguineus*, *Ixodes ricinus* and *Dermacentor reticulatus*. A total of 228 *R. sanguineus* (72.15%), 87 of *I. ricinus* (27.53%) and one *D. reticulatus* (0.32%) samples were collected. Only two dogs had mixed infestation, one included both *I. ricinus* and *R. sanguineus*, and the other included both *I. ricinus* and *D. reticulatus*. Within the *R. sanguineus* species all three developmental stages were present - larvae, nymphs and adults (males and females) and within *I. ricinus* nymphs and adults (males and females). The distribution of developmental stages within species of collected ticks is presented in Table 1.

Table 1. Distribution of developmental stages within every tick species collected from dogs

Tick species	Total 316	Developmental stages			
		female (%)	male (%)	nymph (%)	larvae (%)
<i>Rhipicephalus sanguineus</i>	228	40 (17.54)	51 (22.37)	130 (57.02)	7 (3.07)
<i>Ixodes ricinus</i>	87	33 (37.93)	44 (50.57)	10 (11.49)	0
<i>Dermacentor reticulatus</i>	1	0	1 (100)	0	0

Out of 316 examined ticks, *C. burnetii* DNA was detected only in the brown dog tick *R. sanguineus*, with a total prevalence of 10.53% (24/228). The highest prevalence among collected ticks was detected in female ticks followed by nymphs and male ticks. In total, 30.00% (12/40) of female ticks, 5.88% (3/51) of male ticks and 6.92% of nymphs (9/130) were positive for *C. burnetii*. Animals with positive ticks harbored from two to 36 ticks in total, and no animal harbored all ticks that were positive for *C. burnetii*. Also, no positive ticks were detected in mixed infestations cases found on two dogs. Positive ticks originated from one male dog (1/11) and six female dogs (6/40). Distribution of positive ticks according to the dog from which they were removed is presented in Table 2.

Table 2. Distribution of ticks (positive and total samples) positive for presence of *Coxiella burnetii* DNA according to the dog from which they were removed

Animal	Developmental stages of ticks			
	Female	Male	Nymph	Larva
Dog 1 - Female	5/5	3/5	0	0
Dog 2 - Male	2/5	0/1	0	0
Dog 3 - Female	2/3	0/1	0	0
Dog 4 - Female	1/1	0/1	0	0
Dog 5 - Female	1/1	0/5	0	0
Dog 6 - Female	1/1	0/1	0	0
Dog 7 - Female	0	0	9/36	0
Positive	12	3	9	0
Total			24	

Amplified specific sequences were visualized on 1% agarose gel as a band of ~687 bp. Amplicons were purified and sequencing was performed in both directions. All analyzed sequences from this study were identical to each other. A representative nucleotide sequence was deposited in GenBank under accession No. MH394636. Nucleotide sequence analysis by BLAST was consistent with *C. burnetii* isolates deposited in GenBank with more than 99% identity for IS1111 gene. Comparisons were also made with the published partial sequences of IS1111 region corresponding to *Coxiella*-like endosymbionts [20], which excluded the presence of endosymbionts in ticks (this study).

DISCUSSION

According to the currently available data and bibliography, there are no studies concerning the presence of *C. burnetii* in ticks parasitizing dogs in Serbia. In fact, only few researches have been conducted so far regarding the presence of *C. burnetii* in ixodid ticks in Serbia. In a previous study, conducted in six localities around Belgrade and one locality in Autonomous Province of Vojvodina, the presence of *C. burnetii* was revealed in questing *I. ricinus* (17/27) and *D. reticulatus* (2/10) ticks collected from vegetation but no ticks collected from animals were tested [21]. The results obtained in the current study showed that *R. sanguineus* ticks parasitizing stray dogs in Belgrade are infected with *C. burnetii* with total prevalence of 10.53% (24/228) and no positive ticks were found among *I. ricinus* and *D. reticulatus* ticks. It is possible that the disagreement of the results in these two studies is related to the origin of the samples. If we consider the fact that in our research tick samples were collected from free-roaming dogs, it is impossible to determine the origin of ticks that were recovered from these animals. Also, a recent study was carried out on ten localities in Serbia, regarding the molecular detection of various tick-borne pathogens in spleen samples of golden jackals, and ticks recovered from these animals. The presence of *C. burnetii* was not established neither in spleen samples or in ticks [22].

In accordance with the results from the current study, research data from some endemic areas indicate the extremely low significance of *I. ricinus* in the maintenance of the *C. burnetii* cycle in nature. For example, after the biggest Q fever epidemic in the last ten years that occurred in the Netherlands, the examination of 1891 questing *I. ricinus* ticks collected from vegetation in highly endemic areas did not show the presence of *C. burnetii*. The authors stated that the risk of acquiring infection from questing *I. ricinus* ticks on vegetation in these endemic areas is negligible [23]. Similar results were obtained in Luxembourg, where the absence of *C. burnetii* in 1500 questing *I. ricinus* ticks collected from vegetation was also recorded [24]. Also, in the studies conducted in endemic areas of southern Germany, which included 666 questing *Dermacentor* spp. adults, the presence of *C. burnetii* was not established [25]. Low prevalence of 3.08% were established in Slovakia and Hungary in questing *I. ricinus* ticks [26]. The studies conducted in the highly endemic areas of Basque County (Spain) also showed

that *I. ricinus* has no significance in the epidemiology of Q fever, although it is the most abundant tick species in the region. Out of 1900 ticks collected from animals, no positive samples were established and the authors came to the conclusion that small rodents have the most important role in maintaining Q fever cycle in this region [27,28].

However, a high prevalence of *C. burnetii* that was established in the brown dog tick parasitizing stray dogs in the current study shows that the brown dog tick, which is primarily a dog parasite, may have a significant role in the cycle of Q fever among stray dogs in Belgrade. There is little data in the literature concerning the presence of *C. burnetii* in ticks collected specifically from stray dogs. In the city of Algiers (Algeria) the presence of *C. burnetii* was established in a spleen sample of one stray dog (1/117) by using molecular methods. However, in ticks removed from the dogs, most of which belonged to the brown dog tick, *C. burnetii* DNA was not detected [29]. Similarly, although human Q fever infection related to the dogs had been previously described in Japan [11], later studies did not show the presence of *C. burnetii* in ticks removed from pet dogs in urban environments [30].

Yet, there are various examples that show the presence of *C. burnetii* in brown dog ticks recovered from different animal species and the significance of this tick species parasitizing animals in the epidemiology of Q fever. In the Canary Islands, which is an endemic area for Q fever, authors examined the presence of *C. burnetii* in wild animals, ticks removed from wild and domestic animals and ticks from vegetation. The interesting fact is, although it is an endemic area, all ticks collected from the vegetation were negative, while the prevalence in ticks collected from animals was as high as 6.1%. The brown dog tick was the most common species of tick found on domestic animals, and one positive sample originated from a domestic dog. The authors suggest that in this area *C. burnetii* circulate in the cycle which involves domestic animals, small rodents, lagomorphs, and ticks [31]. Whereas in central Spain the presence of *C. burnetii* was detected in both questing ticks (7.7%) and in ticks collected from domestic and wild animals (3.4%). All positive *R. sanguineus* samples originated from animals with a prevalence of 1.4% (2/146), out of which as many as 106 samples originated from pets. All *R. sanguineus* recovered from dogs were negative, which was explained by the fact that dogs originated from urban environments where Q fever wasn't present [32]. Among the 1485 adult ticks removed from domestic and wild mammals in Sardinia, seven species were identified and most *R. sanguineus* tick originated from dogs (92.3%). Out of 209 *R. sanguineus* pools, four positive were from dogs, and one from a goat [33]. Presence of *C. burnetii* was also established in *R. sanguineus* ticks collected from small ruminants in endemic areas in Cyprus [34] and further studies in Cyprus showed a significant correlation between seropositive ruminants and positive *R. sanguineus* and *Hyalomma* spp. ticks parasitizing them [35]. Recent studies conducted in Cyprus showed the presence of *C. burnetii* in ticks from wild animals on the entire island. Ten tick species were detected, and 28.7% of pooled samples (56/195) were positive for *C. burnetii*. Nine out of 15 *R. sanguineus* pools were positive for *C. burnetii*.

Thus the authors underlined the importance of ticks in the epidemiology of Q fever on the whole island [36].

The high prevalence of *C. burnetii* in the brown dog tick obtained in this study is also significant because of endophilic behavior of this tick species and its ability of surviving inside premises (homes, kennels, animal shelters) where it can complete an entire life cycle in 2-3 months [37]. The transition and feeding of *R. sanguineus* males between dogs in cohabitation was previously described [38]. This type of behavior also accelerates transmission of pathogens during the next feeding [37,38]. This means that if the tick was introduced to the household on the dog, there would be a risk of acquiring infection for both dogs in cohabitation as well as for humans. In addition to the role of ticks as biological vectors for *C. burnetii*, risk analysis should consider the fact that ticks can excrete large amounts of *C. burnetii* in feces, creating an additional risk of infection due to the contamination of the animal's hair and the environment [14]. The presence of dog as a host is necessary in order to maintain a large population of *R. sanguineus* ticks, but cases of brown tick parasitizing humans are described more often [39-41]. The role of ticks in human cases of Q fever is considered negligible, because of the existence of other more common routes of infection. However, there are cases of human infections in the literature, connected with the tick bite, that indicate the possibility of humans acquiring infection this way [42-44].

CONCLUSION

The results obtained in this study place stray dogs into the group of animals that have an epidemiological significance in relation to Q fever in Serbia. The high prevalence of *C. burnetii* in *R. sanguineus*, which is primarily a dog tick, indicates that the importance of dogs in the epidemiology of Q fever is much higher than it was expected. Further studies, which would include the detection of the pathogen in dogs, as well as serological response due to the Q fever agent, are necessary because they would provide a more detailed information about dogs as sentinel species for Q fever in Serbia. Due to the high prevalence of pathogens in ticks that primarily parasitize dogs, it is important, for pet owners, to point out the additional importance of protecting pets from ectoparasites, and for veterinarians to consider Q fever in differential diagnostics, especially in cases of abortion in bitches and early death of pups. Considering that examined animals were stray dogs, further management of these animals is necessary in order to protect their health and welfare.

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Authors' contributions

DB and NS performed molecular and parasitological study, data analysis and wrote the manuscript; DB, NS, and DM carried out the molecular genetic studies; VM collected the samples and assisted with the result analysis; KSD did the sequence analysis, critically revised the manuscript and helped to draft the manuscript; ST helped in the result interpretation, critically revised the manuscript; SR conceived and designed the study and together with ZK helped in the result interpretation, critically revised the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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MOLEKULARNI DOKAZ PRISUSTVA UZROČNIKA KJU GROZNICE *COXIELLA BURNETII* U IKSODIDNIM KRPELJIMA SAKUPLJENIM SA NEVLASNIČKIH PASA U BEOGRADU (SRBIJA)

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Kju groznica je zoonozno oboljenje koje izaziva *Coxiella burnetii*, gram-negativni kokobacil, čije je prisustvo zabeleženo kod velikog broja životinjskih vrsta, prvenstveno domaćih preživara, ali i kod divljih sisara, kućnih ljubimaca, ptica, reptila, artropoda (naročito krpelja) i ljudi. Iako se kontakt sa domaćim životinjama u ruralnim sredinama smatra najčešćim uzrokom oboljenja ljudi, skorija istraživanja su pokazala da je uloga kućnih ljubimaca u epidemiologiji Kju groznice značajno porasla. Iako je primarni put infekcije inhalacija, pretpostavlja se da među životinjama infekcija cirkuliše preko krpelja i da su krpelji odgovorni za heterospecijsko prenošenje, kao i za prostorno širenje uzročnika među kičmenjacima. Cilj ovog istraživanja je bio da se ustanovi prisustvo i prevalencija *C. burnetii* u krpeljima poreklom sa pasa lualica, kao i da se ispita rasprostranjenost vrsta krpelja koje parazitiraju na psima na teritoriji Beograda. Za otkrivanje DNK *C. burnetii* u krpeljima korišćen je PCR protokol koji cilja IS1111 ponavljajući region sličan transpozonu, a rezultati su potvrđeni analizom nukleotidnih sekvenci. Sakupljeno je ukupno 316 krpelja sa 51 psa lualice – 40 ženki (78.43%) i 11 mužjaka (21.57%). Identifikovane su tri vrste krpelja: *Rhipicephalus sanguineus* (72.15%), *Ixodes ricinus* (27.53%) i *Dermacentor reticulatus* (0.32%). Od ukupno 316 ispitanih krpelja, DNK *C. burnetii* je otkrivena samo u braon psećem krpelju *R. sanguineus*, sa ukupnom prevalencijom 10.53% (24/228). Visoka prevalencija *C. burnetii* u *R. sanguineus*, koji je primarno krpelj pasa, ukazuje na značaj pasa u epidemiologiji Kju groznice na teritoriji Beograda.