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Genotyping of *Coxiella burnetii* in Ticks Collected from Wildlife in Greece

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Abstract

Coxiella burnetii is a zoonotic pathogen that causes Q fever in humans and coxiellosis in animals. Ticks are known to be the main vector for *Coxiella* transmission among wildlife and domestic animals, and they can also act as potential reservoirs. This study aimed to screen ticks collected from wildlife in Greece for the presence of *C. burnetii* and to evaluate its genetic diversity using the multi-spacer sequence typing (MST) method. A total of 177 live-feeding ticks were collected from 42 different wildlife animals, including birds, reptiles, and mammals. Molecular identification of the tick species was conducted through amplification and sequencing of 12S rDNA gene, revealing that the majority of the ticks belonged to *Hyalomma aegyptium* species (n=141, 80%). All ticks were tested by qPCR for the *IS1111* gene of *C. burnetii* and 42 (23.7%) were found positive. Among these, 40 (95%) were *H. aegyptium,* with single cases detected in *Haemaphysalis erinacei* and *Rhipicephalus secundus*. Furthermore, MST genotyping of *C. burnetii* was performed on *IS1111*-positive ticks with higher DNA concentrations. Of these, four *H. aegyptium* ticks were successfully amplified and sequenced, and *C. burnetii* was identified as belonging to MST7 genotype group. Overall, this study provides the first documented evidence of *C. burnetii* detection in ticks collected from wildlife in Greece.

Keywords: *Coxiella burnetii*; Wildlife; Ticks; MST; Greece

Introduction

Coxiella burnetii is a gram-negative obligate intracellular bacterium that causes Q fever. The primary mode of disease transmission is through inhalation of contaminated aerosols or dust containing the bacteria, which may originate from the excretions, decidua, and milk of infected animals. Although ruminants are considered the main source of infection for humans, *C. burnetii* reservoirs include a number of wild and domestic mammals, as well as birds and reptiles [1, 2]. Ticks are known as one of the most important vectors of zoonotic pathogens. *Coxiella* infection in ticks has been reported worldwide and has been detected in more than 40 different hard tick species and 14 soft tick species collected from diverse habitats, including vegetation, domestic animals, and wildlife [2-5]. Ticks can act as potential reservoirs by transmitting *Coxiella* both transovarially and transstadially to their offspring [1]. Moreover, infected ticks excrete large amounts of the bacteria in their feces (up to 10^{10} organisms per gram of feces), contaminating the skin of host animals and playing a significant role in the environmental spread of *C. burnetii* [2, 5]. In an *in vitro* blood-feeding experiment, Korner et al. demonstrated the presence of *C. burnetii* excretion in the feces of both *Ixodes*

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ricinus and *Dermacentor marginatus* ticks [6]. Although several studies have indicated that ticks pose a potential risk for coxiellosis in livestock, the transmission of the pathogen to humans through tick bites remains unclear and it seems that ticks have a more critical role as reservoirs rather than as a direct vector of the pathogen. Additionally, ticks seem to have a substantial influence on sustaining the natural cycle of *C. burnetii* infection in wildlife animals [2].

Besides the number of seroprevalence studies conducted in Greece to date, reports of *Coxiella* infection in both humans and domestic animals have also been documented [7-9]. However, there are limited studies on tick infections in Greece, which primarily involve ticks collected from domestic animals. In particular, two separate studies conducted by Chaligiannis et al. and Psaroulaki et al. identified the following ticks infected with *C. burnetii*: *Dermacentor marginatus* collected from goats and sheep, *Haemaphysalis parva* from goats, *Haemaphysalis sulcata* from goats and sheep, *Hyalomma marginatum marginatum* from sheep, *Ixodes gibbosus* from sheep, *Rhipicephalus sanguineus* from goats and dogs, and *Rhipicephalus turanicus* from cattle, sheep, and goats [10, 11].Furthermore, despite the importance of surveillance for *Coxiella* in ticks feeding on wildlife, there is a lack of studies on the genotypic diversity of circulating strains across Greece. The only available data indicate *C. burnetii* MST32 genotype circulates in sheep and goat across eight different areas of Northern Greece, while MST18 and MST65 genotypes have been isolated from patients with Q fever [9, 12, 13]. The aim of the present study was to investigate the presence of *C. burnetii* in ticks infesting wildlife in Greece and to identify the circulating genotypes using Multispacer Sequence Typing (MST).

Materials and Methods

Collection of samples

During the spring/summer of 2024, ticks were collected from wild species living in different areas across Greece. The wild animals from which the ticks were collected had been admitted for treatment to the "Exotic and Wildlife Unit" of the School of Veterinary Medicine at Aristotle University of Thessaloniki, and the "ANIMA-Hellenic Wildlife Rehabilitation Center" from various regions across Greece. All animals admitted for rehabilitation due to various conditions (e.g., injuries, dehydration, emaciation) were documented in an electronic database, along with detailed information such as the animal's age, sex, and origin. Ticks were manually collected after the animal's condition was stabilized, then stored at -20°C. Later they were transferred to the Hellenic Pasteur Institute, where they were kept at -80°C until further analysis.

DNA extraction and PCR amplification

Before DNA extraction, each tick specimen was rinsed twice with distilled water to remove any impurities. Once dried, each sample was cut vertically in half using sterile blades. One half of the body was homogenized manually using sterile mortars and pestles. The homogenized ticks were incubated overnight at 56°C and digested with proteinase K and lysis buffer. Total DNA was extracted using an automated extraction kit (MagCore, RBC Bioscience New Taipei city, Taiwan), using the MagCore® Genomic DNA Tissue Kit, according to the manufacturer's recommendations. All the samples were quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher scientific). DNA from each tick sample was stored at −20°C under sterile conditions to avoid contamination until it was used for polymerase chain reaction (PCR). Molecular identification of the tick species was conducted through amplification and sequencing of 12S rDNA gene. All samples were screened by a TaqMan based real time PCR for the detection of *C. burnetii* targeting a transposon-like *IS1111* region, as previously described [14]. *C. burnetii* DNA was used as a positive control, and nuclease-free water was used as a negative control.

Multispacer sequence typing (MST) and phylogenetic analysis

We selected *IS1111*-positive ticks with Cycle threshold (Ct) values lower than 30 (Ct≤30), which were further processed for MST genotyping using ten different variable spacers (Cox2, Cox5, Cox18, Cox20, Cox22, Cox37, Cox51, Cox56, Cox57 and Cox61). PCR conditions and primers have been previously described [13]. Briefly, each PCR product was electrophoresed using QIAxcel Advanced system (Qiagen) to confirm that the desired bands were present. Purifications were carried out using the QIAquick Spin PCR Purification Kit (Qiagen) according to manufacturer's recommendations. Sequencing data were initially assessed using Chromas software and then were concatenated and aligned with CLUSTAL W program. Sequences obtained were compared with those already available at the MST database (https://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/blast.html accessed on 20 August 2024) to determine the corresponding genotype. Phylogenetic relationships between the MST genotypes were inferred using MEGA11 software by constructing a neighbor-joining tree.

Results

Tick infestations

A total of 177 ticks were removed from 42 different wildlife animals and were included in this study. Specifically, 31 ticks were collected from 6 tortoises *Testudo graeca*, two ticks from one tortoise *Testudo hermanni*, 46 ticks from 19 tortoises *Testudo marginata*, one tick from one fox *Canis vulpes*, five ticks from one jackal *Canis aureus*, seven ticks from one deer *Capreolus capreolus*, 54 ticks from six hedgehog *Erinaceus europaeus*, three ticks from one brown

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hare *Lepus europaeus*, one tick from one little owl *Athene noctua*, six ticks from one common buzzard *Buteo buteo*, one tick from one stock pigeon *Columba oenas*, one tick from one common magpie *Pica pica*, one tick from one collared dove *Streptopelia decaocto*, and eighteen ticks from one barn owl *Tyto alba*. Sequencing of the 12S rRNA gene revealed that the majority of ticks were *Hyalomma aegyptium* (n=141, 80%) followed by *Hyalomma marginatum* (n=18, 10%), *Rhipicephalus secundus* (n=8, 4.5%), *Haemaphysalis erinacei* (n=5, 2.8%), *Hyalomma anatoliticum* (n=3, 1.7%), *Ixodes frontalis* (n=1, 0.5%) and *Ixodes ventalloi* (n=1, 0.5%) (Table 1). Interestingly, *Hyalomma aegyptium* was the only tick species collected from hosts across all tested families, including birds, mammals, and reptiles.

Detection of *C. burnetii* by qPCR

All ticks were tested by qPCR for the *IS1111* gene of *C. burnetii*, with 42 (23.7%) testing positive. *H. aegyptium* was the most commonly infected tick species by *C. burnetii* (n= 40, 95%), followed by single cases of *Ha. erinacei* and *R. secundus* infection. Specifically, 42 out of 177 tick samples (23.7%) tested positive for the pathogen, including 27 *H. aegyptium* (27/46, 59%) obtained from 19 *T. marginatum*, five *H. aegyptium* (5/31, 16%) from six *T. graeca*, four *H. aegyptium* (4/6, 67%) from one *B. buteo*, two *H. aegyptium* (2/53, 4%) from *E. europaeus*, one *H. aegyptium* from one *A. noctua* (1/1, 100%), one *H. aegyptium* (1/2, 50%) from one *T. hermani*, one *Ha. erinacei* (1/5, 20%) from one *C. aureus*, and one *R. secundus* from one *E. europaeus* (1/1, 100%) (Table 1).

MST genotyping

MST genotyping was performed on 13 out of 22 *IS1111*-positive ticks, which had the higher DNA concentration. From these, four were successfully amplified and sequenced for all ten spacers. Two samples were from *T. marginata*, one from *E. europaeus*, and one from *A. noctua*. All four ticks belonged to the species *H. aegyptium*. Sequencing analysis of the spacers from the four samples showed that the spacers Cox2-Cox5-Cox18-Cox20-Cox22-Cox37-Cox51-Cox56-Cox57-Cox61 exhibited the same combination of allele numbers, 4-6-3-5-6-5-8-2-5-6, respectively in all the samples. The detected genotype directly matched a single genotype from the MST database, belonging to MST7. A phylogenetic tree was constructed using MEGA v.11 software. The construction was performed using Neighbor-joining method with 1000 replicates. Analysis of the phylogenetic tree revealed that this genotype has a close relationship with the genotype of MST6, which has been previously detected in ticks originating from Senegal and in a patient from France (Figure 1) [15].

Discussion

Coxiella burnetii is capable of infecting a wide variety of vertebrate and invertebrate hosts, such as both wild and domestic mammals, birds, and arthropods. To date, several studies have reported various tick species carrying *C. burnetii* [2-5]. Majority of these ticks belong to the genera *Amblyomma, Hyalomma* and *Rhipicephalus,* with fewer reports on other genera such as *Dermacentor*, *Haemaphysalis, Ixodes,* and *Ornithodoros* [5]. Particularly,

Table 1: Tick species collected from wildlife and presence of *C. burnetii.*

Family	Host (N)	Tick species (N)	*C. burnetii*	MST genotype (N)
Birds	*Athene noctua* (1)	*Hyalomma aegyptium* (1)	1	7 (1)
	Buteo buteo (1)	*Hyalomma aegyptium* (6)	4	-
	Columba oenas (1)	*Hyalomma aegyptium* (1)	-	-
	Pica pica (1)	*Ixodes frontalis* (1)	-	-
	Streptopelia decaocto (1)	*Hyalomma aegyptium* (1)	-	-
	Tyto alba (1)	*Hyalomma marginatum* (18)	-	-
Mammals	*Canis aureus* (1)	*Haemaphysalis erinacei* (5)	1	-
	Canis vulpes (1)	*Ixodes ventalloi* (1)	-	-
	Capreolus capreolus (1)	*Rhipicephalus secundus* (7)	-	-
	Erinaceus europaeus (6)	*Hyalomma aegyptium* (53)	2	7 (1)
		Rhipicephalus secundus (1)	1	-
	Lepus europaeus (1)	*Hyalomma anatolicum* (3)	-	-
Reptiles	*Testudo graeca (6)*	*Hyalomma aegyptium* (31)	5	-
	Testudo hermanni (1)	*Hyalomma aegyptium* (2)	1	-
	Testudo marginata (19)	*Hyalomma aegyptium* (46)	27	7 (2)
Total	42	177	42	4

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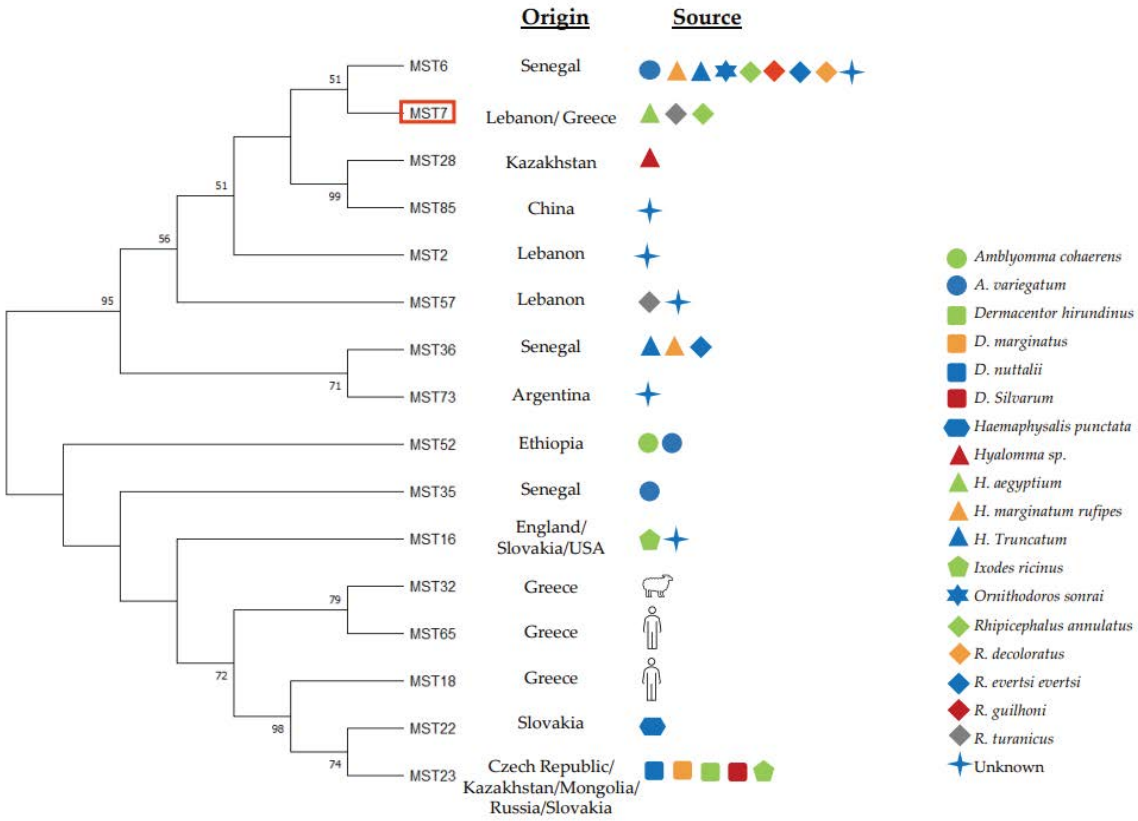

Figure 1: Phylogenetic tree representing the relationships among the different MST groups. The phylogenetic tree was constructed using *C. burnetii* sequences detected in tick samples from around the world, as well as sequences isolated in Greece (Bootstrap values > 50 are shown). The MST32 genotype was detected in a sheep abortion sample, while the MST18 and MST65 genotypes were detected in human samples in Greece.

in Europe, *C. burnetii* has been reported in species of the genera *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus*, with the most commonly detected tick species being *I. ricinus*, *H. marginatum,* and *R. bursa* [4].

In Greece, the first study to identify tick species parasitizing domestic animals date back to collections made between 1983 and 1986 [16]. Since then, several studies have identified a total of 26 hard tick species and subspecies collected from domestic animals, wildlife and humans [17]. Of these, nine belong to the genus *Hyalomma*, and five each belong to the genera *Haemaphysalis*, *Ixodes* and *Rhipicephalus*. Notably, the majority of these tick species are known carriers of pathogens. Among these studies, *C. burnetii* has not been detected in *Hyalomma* species but has been found in other tick species collected from domestic animals, dogs, cats, and humans. On the other hand, limited studies have focused on ticks collected from wildlife in Greece, and none of these have detected the pathogen [17].

Urbanization has significantly increased interactions between humans, domestic animals, and wildlife. This close contact is one of the reasons that wildlife species are considered key players in the epidemiology of zoonotic pathogens, including those transmitted by vectors. In the present study, *C. burnetii* was detected in *H. aegyptium*, *Ha. erinacei*, and *R. secundus* ticks, which were mainly collected from tortoises, with a few collected from other wild animals. This finding aligns with previous reports, which have detected *C. burnetii* in *H. aegyptium* ticks collected from tortoises [18, 19]. The species *H. aegyptium* is known to be the dominant tick found on tortoises of the genus *Testudo* in the Balkans and Mediterranean region [19-21].

We also detected *C. burnetii* in *H. aegyptium* ticks collected from wild birds (*A. noctua* and *B. buteo*) and hedgehogs (*E. europaeus*) as well as in single *R. secundus* collected from a hedgehog and in a *Ha. erinacei* collected from a jackal. To the best of our knowledge, this is the first report of *C. burnetii* in *H. aegyptium* ticks from these hosts. Gong et al. had previously reported presence of *C. burnetii* in hedgehogs, suggesting that these animals and their associated ectoparasites could serve as reservoirs for this pathogen [22]. Moreover *C. burnetii* has been detected in *H. excavatum* ticks collected from hedgehogs in Egypt, *Ha. fava* ticks from hedgehogs in Central China, and *Ha. erinacei* ticks from

hedgehogs in Algeria [23]. Furthermore, *Coxiella* infection in birds was first documented in 1952, with evidence suggesting that various bird species can become infected with this pathogen and excrete it through their droppings. This supports the potential role of avian populations in the epidemiology of *C. burnetii*. To date, several avian wildlife species and migratory birds have been reported to carry *Coxiella*-infected ticks, contributing to the pathogen's spread [24]. In Greece, rickettsiae have been detected in ticks collected from wild birds but no reports exist of *Coxiella*-infected ticks collected from wild birds [25]. While *C. burnetii* has been detected in other tick species parasitizing wild birds and hedgehogs, the identification of this pathogen in *H. aegyptium* collected from these hosts broadens the understanding of its ecological range and host associations [26-28]. Wild birds, in particular, are known to play a vital role in the long-distance dispersal of ticks and tick-borne pathogens due to their migratory behavior [25]. Similarly, hedgehogs, often found in urban and peri-urban settings, serve as important reservoirs, facilitating the spillover of zoonotic agents between wildlife and domestic animals [23].

The molecular characterization of *C. burnetii* is a valuable tool for investigating genotypic diversity among bacterial strains and elucidating their phenotypic heterogeneity [29]. Although various methods have been proposed for *C. burnetii* genotyping, multilocus sequence typing (MST) and multiple-locus variable-number tandem-repeat analysis (MLVA) are considered the most discriminatory and widely used techniques [30]. In particular, MST was introduced by Glazunova et al., who identified 10 non-coding spacers inserted among open reading frames (ORFs), as non-coding DNA regions are more variable than coding sequences due to the absence of selective pressure [13]. This method is mainly used for epidemiological purposes, as it has effectively linked STs to clinical manifestations and strains epidemiology [31]. For instance, MST17 has been associated with severe forms of the disease and has the highest prevalence in cases of community-acquired pneumonia worldwide [30, 32]. Similarly, MST33 was identified as the genotype responsible for the largest recorded Q fever outbreak, which originated in Germany and France before spreading to the Netherlands [33, 34]. Moreover, MST has also been described as a "genotyping method" due to its ability to correlate specific sequence types with their geographical distribution [30]. For example, MST23 has been identified in ticks, animals and humans within a specific area of Eastern Europe and parts of Asia, whereas MST16 exhibits a more worldwide distribution [31]. In addition to MLVA and MST typing, the use of plasmid characterization method has also been proposed as a way to further elucidate the origin of some previously described genotypes [12].

Our analysis revealed the presence of MST7 in all successfully sequenced samples. Due to insufficient DNA quantities, we obtained genotypes for only four ticks, all identified as *H. aegyptium*. As there are no previous reports of this specific sequence type in Greece, we compared our findings with similar studies conducted worldwide. According to the database (http://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/_strains.html), MST7 was previously detected in human blood samples from two patients in Leningrad, Russia, between 1955 and 1957 [13]. Additionally, the same genotype was identified in France in the early 1990s in the heart valve of a patient with the chronic form of the disease [13]. However, there is no substantial evidence or comprehensive studies supporting a correlation between MST7 and specific clinical manifestations. More recently, a study conducted in Lebanon also reported the presence of MST7, identifying *R. annulatus* and *R. turanicus* as the tick species positive for *C. burnetii* (4/15, 27%) [35].

Conclusion

Several studies have reported the presence of *Coxiella burnetii* in ticks collected from domestic animals in Greece. However, to the best of our knowledge, this study provides the first documented evidence of *C. burnetii* detection in ticks collected from wildlife in our country. Molecular analysis confirmed the presence of *C. burnetii* in *H. aegyptium* ticks parasitizing tortoises, hedgehogs, a jackal, a pigeon, and a hawk. MST analysis identified MST7 as the only genotype circulating among the examined ticks. Given the role of ticks as potential reservoirs and vectors of *C. burnetii*, continuous surveillance and monitoring are critical for understanding pathogen transmission dynamics and implementing effective control strategies.

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Institutional Review Board Statement

For the purposes of this study, all the rules and criteria of ethics and bioethics governing the approval and operation of recognized, approved and licensed by the State, Wildlife Rehabilitation Centers and First Aid Stations, were applied in accordance with the legislative provisions of no. YPEN/DDD/88658/2929/2022 Joint Ministerial Decision of the Ministries of Environment, Energy, Rural Development and Food. The ticks collected for the research purpose of this study came from wild animals that were temporarily housed in approved and licensed care centers that meet the technical, veterinary and environmental criteria of the above decision with the aim of their safe and sustainable rehabilitation into the natural environment.

Data Availability Statement

The original contributions presented in the study are

included in the article, and further inquiries can be directed to the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest.

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