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Bacterial pathogens and endosymbionts in ticks

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ABSTRACT

Ticks collected from goats in northern Greece were tested for the presence of tick-borne bacteria. Among adult ticks, 37 (57.8%) were *Rhipicephalus bursa*, 11 (17.2%) *Dermacentor marginatus,* 10 (15.6%) *Ixodes ricinus*, 3 (4.7%) *Rhipicephalus sanguineus* sensu lato and 2 (3.1%) *Haemaphysalis parva*; one (1.6%) *Rhipicephalus* spp. tick was nymph. *Rickettsia monacensis*, *Rickettsia massilae*, *Anaplasma phagocytophilum* and *Anaplasma platys* were detected in *I. ricinus* and *Rh. bursa* ticks. A variety of *Coxiella*-like endosymbionts were detected in all tick genera tested, forming distinct clades from *Coxiella burnetii* in the phylogenetic tree based on the 16S rRNA gene. An additional endosymbiont, *Candidatus* Midichloria mitochondrii, was detected in most of the *I. ricinus* ticks. Surveillance for human pathogens in ticks provides knowledge helpful for the public health, while further studies are needed to determine the role of endosymbionts in tick physiology, vector competence and probably in public health.

1. Introduction

Ticks (class *Arachnida,* order *Ixodida*) are the most important vectors of veterinary pathogens and the second (to mosquitoes) most important vectors of human pathogens since they can transmit a variety of bacteria, viruses, and parasites (de la Fuente et al., 2008). Greece is a Mediterranean country where several tick species have been identified. The main bacterial tick-borne human zoonoses reported so far in Greece include rickettsioses, Q fever (caused by *Coxiella burnetii*) and human granulocytic anaplasmosis (HGA, caused by *Anaplasma phagocytophilum*). Four tick-borne *Rickettsiae* have been identified in humans: *R. conorii* (main agent of Mediterranean spotted fever), *R. aeschlimannii*, *R. sibirica mongolotimonae*, and *R. slovaca*, while *R. massiliae* and *R. rhipicephali* were identified only in ticks (Babalis et al., 1994; Psaroulaki et al., 2005a,b, 2006; Papa et al., 2010; Germanakis et al., 2013; Kostopoulou et al., 2016). Five *Rickettsiae* species were identified recently in human–parasitizing ticks, *R. aeschlimannii, R. massilae*, *R. africae*, *R. monacensis*, and *Candidatus R. barbariae* (Papa et al., 2016), expanding the number of tick-borne *Rickettsia* species circulating in Greece to nine. Q fever and HGA cases have been also reported (Chochlakis et al., 2010, 2009; Psaroulaki et al., 2008; Kokkini et al., 2009), while *A. phagocytophilum* and *C. burnetii* have been detected in ticks (Kachrimanidou et al., 2011; Psaroulaki et al., 2014, 2006). Regarding tick-borne bacterial animal infections, anaplasmosis, canine ehrlichiosis and piroplasmosis are common (Giadinis et al., 2011, 2012; Psaroulaki et al., 2009; Siarkou et al., 2007; Mylonakis et al., 2010; Komnenou et al., 2007; Kontos et al., 1991).

Besides the tick-borne bacterial pathogens, several species of endosymbionts have been described recently, like *Coxiella-*, *Francisella-* and *Rickettsia*-like bacteria (Noda et al., 1997). Derived from the Greek words “endo” (inside), “syn” (together) and “bios” (life), the term endosymbiosis means “live together inside”. Endosymbionts are obligate intracellular organisms, vertically transmitted in ticks, and they have not been isolated in pure culture. The 16S rRNA gene of *Coxiella*-like endosymbionts (CLEs) share some degree of identity (91–98%) with *C. burnetii*, but they form distinct clades in the cluster of the genus *Coxiella* (Zhong, 2012). A more recent study using multi-locus typing showed that all known *C. burnetii* strains originate within a group of CLEs and are the descendants of a *Coxiella*-like progenitor hosted by ticks (Duron et al., 2015).

Candidatus Midichloria mitochondrii (CMM) is an additional symbiotic bacterium harbored by *I. ricinus* ticks. A unique feature of CMM is its intramitochondrial lifestyle and the fact that humans parasitized by *I. ricinus* are seropositive to CMM, prompting the question whether it is a novel pathogen or just a marker of tick bite (Sassera et al., 2006; Mariconti et al., 2012). The role of endosymbionts is not clarified yet, but it has been suggested that they contribute to the fitness of the tick including nutrient pro-

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viding and host defense. Ticks treated with antibiotics that affect *Coxiella* bacteria were shown to lose their fitness based on observations of delayed oviposition, decreased numbers of hatched ticks, decreased numbers of larvae per tick, and the decreased size of ticks treated with antibiotics (Zhong et al., 2007).

The aim of this pilot study was to test ticks collected from goats in northern Greece for the presence of bacterial microorganisms.

2. Materials and methods

2.1. Tick collection

During February 2015 to May 2016 ticks were collected from goats in 8 locations in 5 prefectures of northern Greece (Thessaloniki, Chalkidiki, Pella, Serres and Kavala). Ticks were identified morphologically under a stereo-microscope using taxonomic keys (Estrada-Peña et al., 2004) and stored at −70 °C until further testing.

2.2. DNA extraction and molecular methods

Ticks were homogenized individually in 300 ul PBS and pools of homogenates derived from 1 to 3 ticks were prepared based on tick species, location and collection date. DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany).

PCR assays were applied for the screening of ticks for the presence of 16S rRNA gene of *Rickettsia* (Christova et al., 2003), *Anaplasma* and *Ehrlichia* (Parola et al., 2000), and for superoxide *dismutase* gene of *Coxiella* (Stein and Raoult, 1992). The *Rickettsia*-positive samples were further tested targeting a 632-bp portion of the *Rickettsia*–specific *ompA* gene (Roux et al., 1996). A commercially available multiplex PCR-based tick-borne bacteria flow chip system (Master Diagnostica, Granada, Spain) was also applied for screening the ticks for additional tick-borne bacteria. This system is capable to detect *Anaplasma, Ehrlichia, Coxiella, Rickettsia*, but also *Borrelia, Bartonella* and *Francisella* species.

2.3. Sequencing and phylogenetic analysis

All PCR products were sequenced in an 3130 ABI Genetic Analyzer (Applied Biosystems, Foster City, CA) and nucleotide sequences were initially compared with respective ones available in GenBank using the National Center for Biotechnology Information (NCBI; Bethesda, MD) Basic Local Alignment Sequence Tool (BLAST) search engine (http://blast.ncbi.nlm.nih.gov/blast.cgi). In order to find out the genetic relationships among the detected CLEs, their 16S rRNA sequences were aligned using CLUSTAL W and phylogenetic analysis was performed applying the maximum likelihood model using MEGA6 (Tamura et al., 2013).

Table 1
Adult ticks included in the study.

Tick species	Ticks		Total (pools)
	Male	Female	
Rh. bursa	23	14	37 (20)
D. marginatus	7	4	11 (6)
I. ricinus	2	8	10 (9)
Rh. sanguineus sensu lato	0	3	3 (2)
H. parva	0	2	2 (2)
Total	32	31	63 (39)

3. Results

In total, 64 ticks were collected from 28 goats. All ticks belonged to the *Ixodidae* family, and all except one were adults; 32 of the 63 (50.8%) adult ticks were male. Four genera were identified: *Rhipicephalus*, *Dermacentor, Ixodes* and *Haemaphysalis*. Specifically, 37 (57.8%) ticks were *Rh. bursa*, 11 (17.2%) *D. marginatus,* 10 (15.6%) *I. ricinus*, 3 (4.7%) *Rh. sanguineus* sensu lato and 2 (3.1%) were *Haemaphysalis parva,* while one (1.6%) was *Rhipicephalus* spp. nymph (Table 1).

Known bacterial pathogens, *Rickettsia* and *Anaplasma*, were detected in 11 ticks collected from 5/28 goats: *R. monacensis* was detected in 6 *I. ricinus* ticks (collected from 3 goats) and *R. massiliae* was detected in one *Rh. bursa* tick; *A. phagocytophilum* was detected in one *I. ricinus* tick, and *A. platys* was detected in 3 *I. ricinus* ticks (collected from one goat) (Table 2). The *Omp*A gene sequences of *R. massiliae* and *R. monacensis* [both members of the spotted fever group (SFG) of the *Rickettsiae* genus] were 100% identical to respective sequences of *R. massiliae* MTU5 (GenBank Accession number CP000683) and *R. monacensis* IrR/Munich (GeneBank Accession number LN794217). The sequence of *A. phagocytophilum* and *A. platys* were 100% identical to respective ones in GenBank (such as the sequences with Accession numbers JN181071 and AY530806, respectively). The genetic difference between the two *Anaplasma* species was 1.6% at the nucleotide level.

Table 2
Bacterial pathogens (A) and endosymbionts (B) in ticks collected from goats in Greece. CLE: *Coxiella*-like endosymbionts; CMM: *Candidatus* Midichloria mitochondrii.

	Bacteria	Tick species	Pools Positive/total (%)	Pools (ticks-sex), location, date
A.	*R. monacensis*	*I. ricinus*	6/9 (66.6)	2 (2F), Serres, Feb 2015 2 (2F), Chalkidiki, Mar 2015 2 (1F, 1M), Thessaloniki, Oct 2015
	R. massiliae	*R. bursa*	1/20 (5)	1 (1M), Thessaloniki, Jun 2015
	A. phagocytophylum	*R. bursa*	1/20 (5)	1 (2F), Thessaloniki, Jul 2015
	A. platys	*I. ricinus*	3/9 (33.3)	3 (3F, 1M), Chalkidiki, Mar 2015
B.	CLE	*D. marginatus*	3/6 (50)	1 (1M), Pella, March 2015 2 (4F), Kavala, May 2015
		Rh. sanguineous	2/2 (100)	1 (2F), Kavala, May 2015 1 (1F), Thessaloniki, May 2016
		Rh. bursa	16/20 (80)	10 (6F, 14M), Kavala, May 2015 1 (1F, 1M), Thessaloniki, June 2015 1 (2F), Thessaloniki, July 2015 4 (4M), Thessaloniki, May 2016
		Rhipicephalus spp. nymph	1/1 (100)	1 (1F), Thessaloniki, May 2016
		I. ricinus	1/9 (11.1)	1 (1F), Serres, April 2015
		H. parva	2/2 (100)	2 (2F), Serres, April 2015
	CMM	*I. ricinus*	5/9 (55.6)	1 (1F), Serres, February 2015 2 (2F), Chalkidiki, March 2015 1 (1F), Thessaloniki, October 2015 1 (1F), Thessaloniki, May 2016