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ORIGINAL ARTICLE

Rickettsia species in human-parasitizing ticks in Greece

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Background: Ticks serve as vectors and reservoirs for a variety of bacterial, viral and protozoan pathogens affecting humans and animals. Unusual increased tick aggressiveness was observed in 2008–2009 in north-eastern Greece. The aim of the study was to check ticks removed from persons during 2009 for infection with *Rickettsia* species.

Methods: A total of 159 ticks were removed from 147 persons who sought medical advice in a hospital. Tick identification was performed morphologically using taxonomic keys. DNA was extracted from each individual tick and a PCR assay targeting the rickettsial outer membrane protein A gene of *Rickettsia* spp. was applied.

Results: Most of the adult ticks (132/153, 86.3%) were *Rhipicephalus sanguineus*. *Rickettsiae* were detected in 23 of the 153 (15.0%) adult ticks. Five *Rickettsiae* species were identified: *R. aeschlimannii, R. africae* (n=6), *R. massilae* (4), *R. monacensis* (1), and *Candidatus R. barbariae* (1). To our knowledge, this is the first report of *R. africae*, *R. monacensis*, and *Candidatus R. barbariae* in Greece.

Conclusions: Several *Rickettsia* species were identified in ticks removed from humans in Greece, including those that are prevalent in northern and southern latitudes.

Keywords: Greece, Rickettsia, Ticks

Introduction

Ticks serve as vectors and reservoirs for a variety of bacterial, viral, and protozoan pathogens affecting humans and animals.[1] Several factors such as climatic and ecological changes, bird migration and animal movements play a role on the tick population and the rate of human exposure to ticks, as well as in the emergence of a tick-borne pathogen in a previously unaffected region. In addition, the massive application of molecular tools, including next generation sequencing, enables the detection and identification of pathogens providing better knowledge about their distribution and evolution.

Several tick-borne *Rickettsia* species have been identified in the Mediterranean countries, including Greece. Mediterranean spotted fever cases caused by *Rickettsia conorii* are observed every year, especially in summer, with the brown dog tick, *Rhipicephalus sanguineus,* being its main vector. Additional *Rickettsia* species, like *R. massiliae*, *R. monacensis*, *R. aeschlimannii* and *R. helvetica,* are considered as etiological agents of Mediterranean spotted fever-like illness in the Mediterranean basin.[2] Four tick-borne *Rickettsiae* have been currently identified in humans in Greece: *R. conorii*, *R. aeschlimannii*, *R. sibirica mongolotimonae* and *R. slovaca*, while *R. massiliae* and *R. rhipicephali* were identified only in ticks.[3–9]

Unusual increased tick aggressiveness was observed in summer 2008 in northeastern Greece resulting in increased number of persons bitten by ticks, and two fatal cases, one caused by *R. conorii* and one by Crimean-Congo hemorrhagic fever virus (the first and only report in Greece).[7,10] As a result, many residents of the region sought medical advice in the emergency departments of the regional hospitals. A study conducted in the two main hospitals (in Alexandroupolis and Komotini) during July–September 2008 showed that the majority of the 537 removed ticks were *Rh. sanguineus* (81.5%), while *Hyalomma marginatum* (the main vector of Crimean-Congo hemorrhagic fever virus) accounted for 5.2%.[11] Similar aggressiveness, although to a lesser extent, was observed during 2009. The aim of the present study was to check ticks removed during 2009

from persons who referred to the University General Hospital of Alexandroupolis for infection with *Rickettsia* species.

Materials and methods

Collection of ticks and morphological identification

During April to October 2009, 147 persons (86 males, 58.5%) aged 0.6–88 years (median age 28 years) addressed the Emergency Department of the University General Hospital of Alexandroupolis, in northeastern Greece, because of tick bites. In total, 159 ticks were removed. The approximate time from the tick bite was 1–24 h (based on the persons' report). Ticks were stored without any preservatives at −20°C until their transport to the laboratory in Thessaloniki where they were stored at −80°C until testing. Tick identification was performed morphologically using taxonomic keys.[12]

Molecular methods

Ticks were homogenized individually in a Thermo FastPrep FP120 Cell Disrupter (LabCommerce Inc., San Jose, CA, USA) and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Ticks were tested for probable infection with *Rickettsia* spp. using a PCR assay targeting the rickettsial outer membrane protein (ompA) gene.[13] PCR products were sequenced in an 3130 ABI Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and nucleotide sequences were compared with those available in GenBank using the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) Basic Local Alignment Sequence Tool (BLAST) search engine (http://blast.ncbi.nlm.nih.gov/blast.cgi).

Statistical methods

Differences between tick species and affected age groups were tested by χ^2, while differences in the number of removed ticks per month were tested by student's t test.

Results

All 159 ticks belonged to the *Ixodidae* family. Six were *Rh. sanguineus* larvae. Among the 153 adult ticks, 139 (90.8%) were female. Seven tick species from four genera were identified. Specifically, 132 were *Rh. sanguineus* (86.3%), eight *Rh. bursa* (5.2%), seven *Rh. turanicus* (4.6%), two *Ixodes ricinus* (1.3%), two *H. marginatum* (1.3%), and one each (0.65%) *H. rufipes* and *Dermacentor marginatus* (Table 1).

Most persons (86/147, 58.5%) were male and eight of them were bitten by more than one tick (2–3). For 123 persons the age was available; 133 ticks were removed from these persons. Most ticks (50/133, 37.6%) were removed from persons belonging to the age group of 0.1–9 years; however, the difference among groups was not significant ($p>0.05$) (Table 2). The number of ticks removed from humans differed significantly among months ($p=0.015$) (range 3–47). Most ticks were removed in August (47/159, 29.6%) and September (43/159, 27%) (Figure 1). A variety of tick species were identified during May, while the ticks removed during summer and autumn were mainly *Rhipicephalus* spp. (Figure 1).

Rickettsia DNA was detected in 23 of the 153 (15%) adult ticks; all larvae were negative. The 23 *Rickettsia* spp. positive ticks were removed from 22 humans (14 males, 63.6%) with a median age of 33 years (range 1.4–88 years). Five *Rickettsia* species were identified: *R. aeschlimannii* (n=11), *R. africae* (6), *R. massilae* (4), *R. monacensis* (1), and *Candidatus R. barbariae* (1) (Table 3). The first four species are associated with human disease, while the pathogenicity of the last one remains to be elucidated.

Specifically, *R. aeschlimannii* was detected in 11/131 (8.4%) *Rh. sanguineus*. Two groups of sequences were obtained, presenting 99.5% and 98.2% identity at nucleotide and amino acid level, respectively. One group consisted of six sequences which were identical to each other and to *R. aeschlimannii* strain TR/Orkun-H.aegyp85/Ankara (accession number JQ691727) obtained from an *H. aegyptium* in Ankara, Turkey[14]; the second group consisted of five sequences which were identical to each other and to *R. aeschlimannii* strain TR/Orkun-H.nymph88/Ankara (accession number JQ691729) obtained from an *Hyalomma* spp. nymph in Ankara, Turkey.[14] *R. africae* was detected in 6/131 (4.6%)

Table 1. Adult ticks removed from humans in Alexandroupolis, northeastern Greece, 2008–2009

	2008[a]			2009		
Tick	Male	Female	Total	Male	Female	Total
Rhipicephalus sanguineus	1	218	219 (94.8)	1	131	132 (86.3)
Rh. bursa	1	3	4 (1.7)	7	1	8 (5.2)
Rh. turanicus	0	1	1 (0.4)	4	3	7 (4.5)
Hyalomma marginatum	4	2	6 (2.6)	1	1	2 (1.3)
H. rufipes	1	0	1 (0.4)	0	1	1 (0.7)
Ixodes ricinus	0	0	0	0	2	2 (1.3)
Dermacentor marginatus	0	0	0	1	0	1 (0.7)
Total	7	224	231 (100)	14	139	153 (100)

[a] Papa et al.[11]

Table 2. Number and species of the ticks removed from 123 humans grouped by age, northeastern Greece, 2009

Tick species	Age groups of humans							
	0–9	10–19	20–29	30–39	40–49	50–59	>60	Total (%)
Rhipicephalus sanguineus	43	9	8	8	12	10	17	107 (80.5)
Rh. bursa	0	0	1	2	1	1	3	8 (6.0)
Rh. turanicus	2	2	0	1	2	0	0	7 (5.3)
Hyalomma marginatum	0	0	0	0	1	1	0	2 (1.5)
H. rufipes	0	0	0	0	0	1	0	1 (0.7)
Ixodes ricinus	0	0	0	0	0	0	1	1 (0.7)
Dermacentor marginatus	0	0	0	0	1	0	0	1 (0.7)
Rh. sanguineus (larvae)	5	0	0	1	0	0	0	6 (4.5)
Total	50	11	9	12	17	13	21	133 (100)

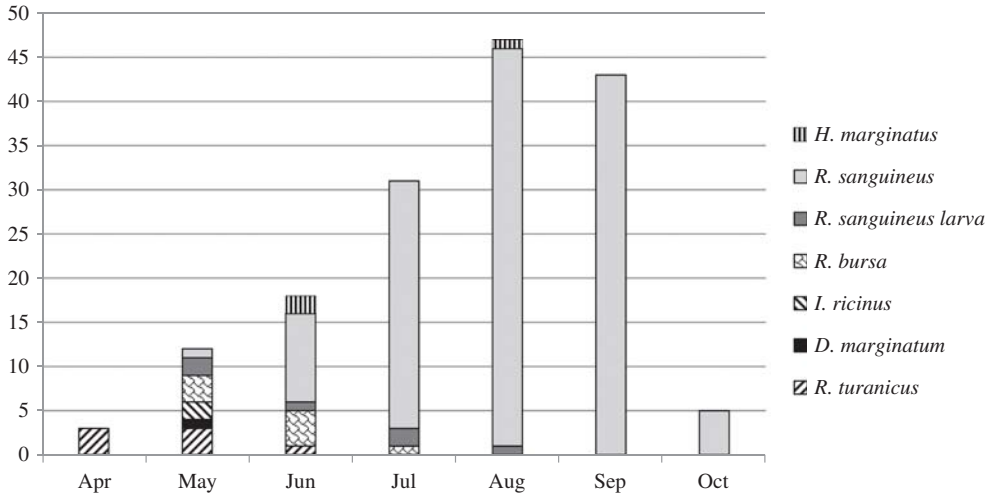

Figure 1. Monthly distribution of human-parasitizing ticks, northern Greece, 2009. *D*: *Dermacentor*; *H*: *Hyalomma*; *I*: *Ixodes*; *R*: *Rhipicephalus*

Table 3. *Rickettsia* (*R.*) species detected in adult ticks removed from humans, in northeastern Greece, 2009

Tick	Female n positive/n tested	Male n positive/n tested	Total n positive/n tested
Rhipicephalus sanguineus	17/131 11 *R. aeschlimannii* 6 *R. africae*	0/1	17/132 (12.9%)
Rh. bursa	0/1	2/7 1 *Candidatus R. barbariae* 1 *R. massiliae*	2/8 (0.25%)
Rh. turanicus	2/3 2 *R. massiliae*	1/4 1 *R. massiliae*	3/7 (42.8%)
Ixodes ricinus	1/2 1 *R. monacensis*	0	1/2 (50.0%)
Dermacentor marginatus	0	0/1	0/1
Hyalomma marginatum	0/1	0/1	0/2
H. rufipes	0/1	0	0/1
Total	20/139 (14.4%)	3/14 (21.4%)	23/153 (15.0%)

Rh. sanguineus: all sequences were identical each other, and identical to the sequence of strain *Rickettsia* sp. UzbekistanHA123S (AB795208) detected in *H. aegyptium* fed on a tortoise (*Tetsudo horsfieldii*) imported to Japan from Uzbekistan,[15] presenting 0.4% nucleotide difference from the respective sequence of strain EgyRickHd-Qalet El-Nakhl-9 (HQ335137) detected in *H. dromedarii* in Egypt.[16] *R. massilae* was detected in 3/7 (42.8%) *Rh. turanicus* and 1/10 (10%) *Rh. bursa*; sequences were identical to the respective sequence of the strain MTU5 (CP000683) isolated from *Rh. turanicus* collected on horses in Camargue, France.[17] *R. monacensis* was detected in 1/3 (33%) *I. ricinus*, being identical to the respective sequence of the type strain IrR Munich isolated from *I. ricinus* collected in a city park in Munich, Germany (KT119437).[18] *Candidatus R. barbariae* was detected in 1/10 (10%) *Rh. bursa*, with sequence identical to a strain detected in *Rh. turanicus* in Sardinia, Italy (EU272186).[19]

Discussion

Due to an unusual tick aggressiveness observed in northeastern Greece during 2008 and 2009, studies were conducted to identify ticks attached to persons living in this region and check them for infection with *Rickettsia* species. Most (132/153, 86.3%) of the adult ticks removed from humans in 2009 were *Rh. sanguineus*. The respective percentage in summer of 2008 in the same hospital was even higher (219/231; 94.8%)[11] (Table 1). This difference is because the 2008 study started in June, following the fatal Crimean-Congo hemorrhagic fever and Mediterranean spotted fever cases, while the present study started earlier, in April, resulting in the collection of additional tick species, like *I. ricinus* and *D. marginatus*, which prefer colder weather. However, it was evident that again *Rh. sanguineus* was almost the only tick species removed from humans from July up to the end of the study in mid-October (Figure 1). A similar study conducted in the urban area of Istanbul showed that among 1054 removed ticks, most were *H. aegyptium* nymphs (50%) and *I. ricinus* female ticks (27%).[20]

Rickettsiae spp. were detected in 15.0% of the adult ticks. Apart from *R. aeschlimannii* and *R. massilae*, which have been detected previously in humans or ticks in Greece, *R. africae*, *R. monacensis,* and *Candidatus R. barbariae* are reported for the first time in the country. However, it has to be mentioned that *R. africae* has been detected in the spring of 2010 in *H. marginatum* larva and nymphs on migratory birds (common redstart, song thrush, and Eurasian siskin) which landed on a Greek island (Antikythira).[21] *Hyalomma* ticks which attach as unfed larvae to migrating birds can remain up to 4 weeks on the same bird and may be transported long distances, e.g., from sub-Saharan Africa to northern Europe.[21] *R. africae* is known to be transmitted by *Amblyomma* ticks in sub-Saharan Africa and the French West Indies causing African tick bite fever characterized by fever and multiple eschars.[22] The detection of *R. africae* in 4.5% (6/132) of the *Rh. sanguineus* ticks attached on humans suggests that this species is already established in Greece.

None of the persons presented any symptoms of rickettsial infection. This can be explained in part by the rapid removal of the ticks (due to increased awareness), preventing infection by avoiding the inoculation of infected tick saliva. The species of the *Rickettsia* is also an important factor for pathogenicity. Especially for *R. aeschlimannii,* only one human case has been reported.[5] In Spain, 35 persons bitten by *R. aeschlimannii*–positive ticks did not present any symptoms of spotted fever and this was attributed to the rapid removal of the ticks.[23] Advanced age and immunocompromised situations are classical risk factors for severe forms of rickettsiosis.[24] In the present study the median age of the persons who were bitten was 26 years (range 1.3–88 years); the oldest person was an 88-year old female bitten by *Rh. bursa* tick in which *Candidatus R. barbariae* was detected. The pathogenicity of this agent is not determined yet.[19] It has been detected also in other countries, like Cyprus, Israel and Portugal (PoTiRb169).[25–27] Additional *Candidatus Rickettsia* spp. have been described in Europe in the last 10 years, and their role for humans remains to be elucidated. A unique property of the pathogenic *Rickettsiae* is their affinity for vascular endothelial cells lining small and medium-sized blood vessels in humans.[28] Not much information is currently available about the host and pathogen factors that enable Rickettsia to cross the species barriers and become capable of causing disease in humans. Even the known pathogenic species can cause asymptomatic infections, while agents previously considered as non human pathogens proved later to cause disease in humans.

The impact of climate on the behavior of the widely distributed *Rh. sanguineus* has been previously reported, when similar tick aggressiveness was observed in 2007 in southern France, where the weather in April was associated with the highest temperatures noted in the region since 1950; the authors predicted that, as a result of global warming, more pathogens transmitted by the brown dog tick may emerge in the future.[29] The higher than usual summer temperatures observed in Greece in 2008 and 2009 (mainly in 2008) might affect the behavior of *Rh. sanguineus*.

The pathogenic potential of *R. massiliae* has been confirmed recently.[30] In Greece, it has been isolated from *R. sanguineus*,[8] while in Cyprus, it was identified in *Rh. turanicus* and *Rh. sanguineus* ticks collected from domestic and wild animals.[26] In the present study it was detected in *Rh. turanicus* and *Rh. bursa* ticks.

H. marginatum ticks (the main vectors of Crimean-Congo hemorrhagic fever virus), accounted for only 1.2% of the ticks removed in 2009 (in 2008 the percentage was 2.6 in the same hospital, and 7.3% in the Hospital of Komotini). No Crimean-Congo hemorrhagic fever case has been reported in Greece apart from the case observed in Komotini in 2008.

Two of the 153 adult ticks, were *I. ricinus*, one infected by *R. monacensis*. This tick species was first isolated in 2002 in Germany,[18] and later in other European countries.[31–33] It is one of the most prevalent species in Europe, especially in relatively humid regions, and is the main vector of *R. monacensis*.

Limitations of the study

The ticks were collected only during April–October. This was because persons started visiting the hospital because of tick bites only in late April; the number of visits peaked in August, and declined sharply in mid October (Figure 1). This can be explained by the high activity of *Rh. sanguineus* ticks observed in the summer months of 2008–2009. Another limitation of the

study was the testing for *Rickettsia* using only one target gene for PCR amplification. This was selected for better identification of the spotted fever group *Rickettsia*. In a previous study using two target genes (17-kDa antigen gene and ompA gene) in a clinical sample both PCRs were positive (*R. conorii*).[7] The fact that *R. conorii* was not detected in the present study is likely due to its pathogenicity for ticks, since it was shown that exposure of *Rh. sanguineus* to *R. conorii* strain Malish produces lasting harmful effects on their survival.[34]

Conclusions

The present study shows that ticks removed from humans in northern Greece harbored several *Rickettsia* species, including those that are prevalent in northern and southern latitudes, like *R. monacensis* and *R. africae*, respectively. Billions of birds, many of them tick-infested, migrate annually between Europe and Africa, with the potential to disseminate several tick-borne pathogens, including *rickettsiae*. This fact, combined with climatic and ecological changes, plays a role in the geographic distribution of the *Rickettsia* species. Further studies in ticks and in patients with symptoms resembling rickettsiosis will enable the better understanding of the epidemiology and pathogenicity of rickettsiae in Greece and worldwide.

Authors' contributions: AP designed the study, evaluated the results and wrote the paper; KX performed the molecular testing; TK analysed the data and contributed in the writing; MP and EM undertook the work in the hospital; SS and IC performed the identification of the ticks; all authors read and approved the manuscript. AP is the guarantor of the paper.

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