Kafkas Univ Vet Fak Derg
19 (1): 19-24, 2013
DOI: 10.9775/kvfd.2012.7033

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

# *Borrelia burgdorferi* s.l. and *Rickettsia* spp. in Ticks Collected from European Part of Turkey

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**Makale Kodu (Article Code): KVFD-2012-7033**

## Summary

This study was performed in ticks collected with the flagging method from localities situated along Anatolian side of Istanbul to the Bulgarian border of Turkey which is under the effect of Black Sea climate. All ticks which were collected seasonally were screened for the presence of *B. burgdorferi* s.l. and *Rickettsia* spp. in pools. As a result, indicated agents were revealed to be common in ticks of studied localities, *Ixodes ricinus* being the predominant tick which was active throughout the year.

***Keywords:*** *Tick, Borrelia burgdorferi s.l., Rickettsia, Turkey*

# Türkiye'nin Avrupa Yakasından Toplanan Kenelerde *Borrelia burgdorferi* s.l. ve *Rickettsia* spp. Varlığı

## Özet

Bu çalışma, İstanbul'un Anadolu yakasından, Bulgaristan sınırına kadar devam eden, Trakya'nın karadeniz iklimi etkisi altında bulunan alanlardan bayraklama yöntemiyle toplanmış kenelerde yürütülmüştür. Mevsimsel düzende toplanmış olan kene havuzlarında *B. burgdorferi* s.l. ve *Rickettsia* spp. varlığı araştırılmıştır. Sonuç olarak, çalışılan alanlarda ilgili etkenlerin yaygın olarak görüldüğü ve yıl boyu aktivite gösterebilen *Ixodes ricinus*'un en baskın kene olduğu anlaşılmıştır.

***Anahtar sözcükler:*** *Kene, Borrelia burgdorferi s.l., Rickettsia, Türkiye*

## INTRODUCTION

Rickettsiosis is a zoonotic disease caused by obligate intracellular gram-negative alphaprotobacteria in the genus *Rickettsia* included in the order Rickettsiales and the family Rickettsiaceae. Belonging to the genus *Rickettsia*, 25 species the pathogenicity of which is certain, and some 20 species with pathogenicity which remains unascertained were reported from the vertebrates. Most of them are included in spotted fever group, and transmitted by ticks. A great number of tick species of different genera are responsible for transmission of one or more rickettsial species [1]. Vector ticks are reservoir for the disease at the same time, but every tick species is not reservoir for every agent [2].

Lyme borreliosis which is caused by *Borrelia burgdorferi* sensu lato (s.l.) complex in the genus *Borrelia*, family Spirochaetaceae, and seen in temperate zone of the northern hemisphere is the most common arthropod-borne disease. *Borrelia burgdorferi* s.l. complex is reported to comprise some 18 genospecies, of which the pathogenities, resulting clinical pictures, and species of vertebrate host and vector ticks are different. In general, vector ticks for transmission of Lyme borreliosis fall within the genus *Ixodes* [3,4].

Ticks are biological or mechanical vectors of many species specific or zoonotic agents in humans and animals. Depending on species and developmental stages, ticks exhibit varying degrees of host selection. However, when they could not quest for their own appropriate host, they

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can use different animals as host. There is not a known tick species which tends to complete its life cycle on humans. On the other hand, from the reported cases to date, we know that 222 tick species, especially 33 of them feed on humans [5,6], and especially some of those display high interest in humans [7,8]. This factor plays an important role in epidemiological characters of tick-transmitted diseases. The data on the tick abundance, frequency of tick-bite cases, and infection prevalence of these ticks in a given region is very significant to determine the related risk for human. This study aims to investigate the presence of *Rickettsia* spp. and *Borrelia burgdorferi* s.l. in ticks collected from some spesific localities in the Thrace region and Istanbul, Turkey seasonally over one year period.

## MATERIAL and METHODS

### *Study Area and Material*

The study covered five localities situated along Anatolian side of Istanbul to the Bulgarian border. These areas were selected as a subsample of the natural biotopes of Black Sea climatic effects *(Fig. 1)*. Deciduous oak forest patches with scattered natural or artificial grasslands, shrub and scarce vegetation which are known to be suitable for *Ixodes ricinus* especially were sampled. Although sampling areas differed based on habitat and structure composition, they had common features. Ticks were collected from two foci (Beykoz and Belgrat Forest) monthly and from other foci twice per season in the year 2009.

The flagging method, slightly modified from Ginsberg and Ewing [9], was used to collect ticks. A white, cotton flannel (1x1 m) was dragged through vegetation for 100 m, by checking every 10 to 20 m. Dragging was conducted five to ten times per sampling site in the morning (8.00 am-11.00 am) and in the evening (5.00 pm-8.00 pm) in order to weaken the effects of changing weather conditions in collection days. All ticks were removed from the flag, preserved in 70% ethanol and transported to the laboratory for identification. Adult ticks were identified to species level, while larval and nymphal stages to genus level with the exception of *Hyalomma aegyptium* nymphs under a stereomicroscope [10-12]. The collected ticks were sorted according to the species, site and season of collection, and stored in 70% ethanol at 4°C until further studies.

### *Pooling of Ticks and DNA Extraction*

Of 194 tick groups, 77 groups were selected based on collection sites, species and developmental stages for DNA extraction. Those groups were pooled as follows: two groups of *D. marginatus* adult (one individual in each group), one group of *H. aegyptium* adult (one individual), eight groups of *H. aegyptium* nymphs (1-4 individuals), six groups of *Haemaphysalis* nymphs (1-5 individuals), 32 groups of *Ixodes* spp. nymphs (1-27 individuals), 23 groups of *I. ricinus* adult (1-25 individuals), one group of *R. bursa* (one individual), four groups of *R. sanguineus* group (1-13 individuals).

Ticks of each pool were decontaminated in 70% ethanol, washed with sterile double distilled water, air-dried for a maximum of 10 min, and then homogenized in liquid nitrogen. DNA was extracted with QIAamp DNA Mini Kit (10) [13], and the obtained samples were stored at -80°C until use.


**Fig 1.** Topographic map of the study area. Stars point out the studied localities (The map was modified from *http://www.arcgis.com*)

**Şekil 1.** Çalışma alanının topografik haritası. Yıldızlar, çalışılan lokaliteleri işaret etmektedir (Harita *http://www.arcgis.com*'dan uyarlanmıştır)

***Semi Nested PCR***

OspA gene of *Borrelia burgdorferi* s.l. (Primers; OSPAFw1: ttgggaataggtctaatattagc, BorR: actaatgttttvccatcttc, OSPAFw2: atttcctggaagcttaatgc, PCR conditions; first step: OSPAFw1-BorR, second step: OSPAFw2 BorR, 94°C/2 min (94°C/1 min, 50°C/1 min, 72°C/2 min) 45 cycles, 72°C/10 min) and citrate synthase (gltA) gene of *Rickettsia* spp. (Primers; RickF1: gggttttggtcatcgtgtat, RickR1: cccgaataaaaatcaacatt, RickR2:tctctcaataaaatattcatc tttaag, PCR conditions; first step: Rick F1-RickR1, second step: Rick F1-RickR2, 95°C/2 min (95°C/30 s, 50°C/1 min, 72°C/1 min) 44 cycles, 72°C/5 min) were targeted [14].

## RESULTS

A total of 2624 ticks were collected within the scope of the study. Given the percentage and developmental stages, the numbers and species of those ticks were as follows: 2343 (89.29%) *Ixodes* spp. (*I. ricinus* 16.35%, nymph 22.70%, larva 60.95%), 126 (4.80%) *Hyalomma* spp. (*H. aegyptium* 3.97%, nymph 23.02%, larva 73.01%), 106 (4.03%) *Rhipicephalus* spp. (*R. sanguineus* gr. 66.98%, *R. bursa* 2.83%, nymph 11.32%, larva 18.87%), 35 (1.34%) *Haemaphysalis* spp. (*H. inermis* 14.29%, nymph 60.0%, larva 25.71%) and 14 (0.54%) *Dermacentor* spp. (*D. marginatus* 100%). Most of the ticks were obtained over the summer (978 ticks, 37.27%), whereas 223 ticks (8.50%), all *Ixodes* spp., were collected during the winter. It was possible to find *Ixodes* spp. at all developmental stages in both summer and winter seasons; however nymphs were more abundant in spring (44.55%) and winter (27.07%), while larvae in summer (48.25%) and fall (45.17%). The data are summerized in *Table 1*.

We found that 60 of 77 pools yielded positive PCR results for *Rickettsia* spp. (%77.92 positive), and 12 pools for *Borrelia burgdorferi* s.l. (15.58% positive). *Table 2* indicates PCR results.

## DISCUSSION

Distribution, density and seasonal activities of tick species depend on vegetation, seasonal parameters and host availability [15]. Forestlands with high rate of precipitation and high humidity (80% at least) are reported to be more suitable for *I. ricinus* especially [6,16]. The preferred study areas which are covered by oak forests are in fact convenient for this tick species; however mean areal values of precipitation (79.0 mm, 75.6 mm, 68.1 mm and 79.2 mm in winter, spring, summer and fall, respectively, as found out from records of Turkish State Meteorological Service) were lower than the averages in the year the study was performed. On the

***Table 1.*** *Tick numbers according to seasonal distribution*
***Tablo 1.*** *Toplanan kene sayılarının mevsimsel dağılımı*

| Ticks | Number of Ticks Collected by Seasons | | | | Total |
|---|---|---|---|---|---|
| | Spring | Summer | Fall | Winter | |
| *Ixodes* spp. | | | | | |
| Larva | 40 | 689 | 645 | 54 | 1428 |
| Nymph | 237 | 34 | 117 | 144 | 532 |
| *I. ricinus* | 219 | 123 | 16 | 25 | 383 |
| *Hyalomma* spp. | | | | | |
| Larva | 57 | 35 | - | - | 92 |
| Nymph | - | 28 | 1 | - | 29 |
| *H. aegyptium* | 4 | 1 | - | - | 5 |
| *Rhipicephalus* spp. | | | | | |
| Larva | - | 20 | - | - | 20 |
| Nymph | 6 | 6 | - | - | 12 |
| R. bursa | 2 | 1 | - | - | 3 |
| *R. sanguineus* gr. | 54 | 17 | - | - | 71 |
| *Haemaphysalis* spp. | | | | | |
| Larva | - | 9 | - | - | 9 |
| Nymph | 8 | 13 | - | - | 21 |
| *H. inermis* | 5 | - | - | - | 5 |
| *Dermacentor* spp. | | | | | |
| *D. marginatus* | 9 | 2 | 3 | - | 14 |
| **Total** | **641** | **978** | **782** | **223** | **2624** |

**Table 2.** *Semi nested PCR results of tick pools*
**Tablo 2.** *Kene havuzlarında uygulanan semi nested PCR sonuçları*

| Tick Species | Counts of Pools | Tick Numbers in the Pools | Number of *Rickettsia* spp. Positive Pools (%) | Number of *B. burgdorferi* sl. Positive Pools (%) |
|---|---|---|---|---|
| *D. marginatus* | 2 | 1 | 1 (50) | - |
| *H. aegyptium* | 1 | 1 | 1 (100) | - |
| *H. aegyptium* nymph | 8 | 1-4 | 5 (62.2) | 1 (12.5) |
| *Haemapysalis* spp. nymph | 6 | 1-5 | 4 (66.7) | - |
| *Ixodes* spp. nymph | 32 | 1-27 | 28 (87.5) | 4 (12.5) |
| *I. ricinus* | 23 | 1-25 | 19 (82.61) | 7 (30.43) |
| *R. bursa* | 1 | 1 | 1 (100) | - |
| *R. sanguineus* gr. | 4 | 1-13 | 1 (25) | - |
| **Total** | **77** | **1-27** | **60 (77.92)** | **12 (15.58)** |

other hand, the preferred study areas are under the effect of Black Sea climate, a proxy for high humidity.

Studies show that *I. ricinus* share humid forested places with species of *Haemaphysalis*, another forest tick. It was reported that *Hyalomma* spp. is a typical open field tick, whereas *Dermacentor* spp. and *Rhipicephalus* spp. have characteristics of both tick genera [17]. Except for *H. inermis*, some *Haemaphysalis* species live in more arid regions [18], and *R. bursa* is found in habitat and has period of activity similar to that of *Hyalomma* spp.[7,19]. Throughout the same region, ratio of adults of human biting *H. marginatum*, *H. aegyptium*, *R. bursa*, *R. sanguineus* gr., *I. ricinus*, *H. inermis*, *H. parva* and *D. marginatus* to each other was 4.8:7.7:6.2:1 2.8:65.3:0.1:1.9:1.2 [7,8]. However, that ratio is 0.0:1.0:0.6:14.8: 79.6:1.0:0.0:2.9 in this study. Considering that the previous studies [7,8] have been conducted on human biting ticks, differences between the ratios may have arisen from the human contact opportunity and other environmental determinants in the collection sites.

*Ixodes ricinus* is active at all times of the year in many regions in Europe. Its adults show peak in spring especially, and the major peak for nymphs occurs in the end of spring, starting up with the beginning of the season. However, emergence of larvae takes place a couple of months after nymphs, although it may change according to region [4,20]. Adults and nymphs were seen in spring in our study as well, and larvae showed a second peak in fall, as confirmed by the study of Randolph et al.[21]. Yet, a 10°C temperature in the environment is reported to be threshold for larvae, and other developmental stages are affected by cold weather, the degree of affection being lower [20]. Seasonal temperature average were 8.2°C, 12.2°C, 23.8°C and 16.8°C in winter, spring, summer and fall, respectively in the study area during the year 2009, and ticks of three developmental stages were found in every season. We could not find any ticks in areas of grassland and scarce vegetation at temperatures below 10°C, but it was possible to collect them from the places covered by oak leaves. Therefore, it was obvious that fallen oak leaves protected ticks against the unfavourable weather conditions at certain level.

Many tick species are known to play role in transmission of the rickettsial agents in the spotted fever group [22,23]. Prevalence of this agent ranges from 1.6% to 67% [24-27] in European populations of *I. ricinus*, and 1% to 84.57% in other tick species [2,28]. In Turkey, almost %50 *Rickettsia* spp. positivity was detected in ticks other than *Ixodes* spp. collected from domesticated animals in different regions [29]. In two serological surveys of people in risk groups from Black Sea [30] and Mediterranean regions [31] of the country, seropositivity was found as 11.7% and 13.7%, respectively. However, rickettsial infections are rarely seen (3-5 annual cases per million), and cases are mostly from coastal provinces and Thrace region, which was sampled in the present study, being in the lead. In addition, *Rickettsia conorii* subsp. *conorii* was detected in clinical cases [32] from Thrace region, which is the only reported species from human cases in the country.

Overall, 77.92% of tick pools tested was found to be positive for *Rickettsia* spp. in the present study, and *Ixodes* spp. nymphs and *I. ricinus* adults showing higher percentages (87.5% and 82.61%, respectively) than the other tick species. Even though the primers used were specific to infectious *Rickettsia* spp.[14], molecular techniques employed in analysis of restricted regions of relevant gene are known to be conflictual, and thus examining of more than one gene region is suggested, especially in case of facultative endo-symbiont *Rickettsia* spp.[27]. On the other hand, importance of spotted fever group rickettsiae in vertebrates is a little known fact [33], although some endosymbiont species are vertically transmitted among the ticks with no involvement of a vertebrate [34]. Detailed molecular analyses indicated that *Rickettsia* spp. is a more complex group than expected [1]. Moreover, although many *Rickettsia* species isolated from ticks have not yet been implicated in human pathology, it was reported that these ricketsiae should be considered as potential pathogens [35,36].

As to studies on the prevalence of *Borrelia burgorferi* s.l. in *I. ricinus* in Europe, positivity was found to be 0-2.9%,

8.9-10.1% and 2-56.3% in larvae, nymphs and adults, respectively [37-40]. From the studies carried out with different methods 4-38.7% positivity was reported in field-collected *I. ricinus* in different regions in Turkey [41-43]. In fact, the relevant agent was detected in *Ixodes spp.* nymphs and adults as 12.5% and 30.4%, respectively and in *H. aegyptium* nymphs as 12.5% in our study. Comparatively, *H. aegyptium* nymphs collected from tortoises in the same study area had a positivity of 14.3% [44]. Nevertheless, Guner et al.[45] reported that agents isolated from tortoise ticks differ from common *B. burgdorferi* s.l. species genetically, and therefore suggested that those agents should be named as *B. turcica*.

Approximately 65.500 Lyme patients are seen annually in Europe [4]. Lyme disease was reported from Turkey for the first time in 1990. While a seropositivity of 44% was detected in risky areas, recorded cases do not pass a couple of dozen [46-48]. Moreover, the disease shows different clinical pictures depending on the agent and the patient, and its symptoms can be confused with a variety of systemic diseases [49].

In conclusion, 1) Contrary to the common belief, some tick species can cause health problems even in winter months in Turkey. 2) Higher *Rickettsia* spp. and *B. burgdorferi* s.l. positivity and tick-attachment rates [7,8] indicate that status of tick-borne diseases in humans can be far worse than previously known. 3) Further studies involving surveillance of tick-borne disease agents are necessary to reveal transmission of pathogens of public health importance.

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