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Seroepidemiology of *Borrelia burgdorferi sensu lato* and *Anaplasma phagocytophilum* in wild mice captured in Northern Turkey

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SUMMARY

An expedition across the Asian part of the Black Sea coast and national parks of Northern Turkey was organized in the summer of 2001 to investigate the presence of *Borrelia burgdorferi sensu lato* (*s.l.*), Lyme borreliosis agent, and *Anaplasma phagocytophilum*, human granulocytic ehrlichiosis agent, in wild mice. A total of 65 *Apodemus flavicollis*, *Apodemus sylvaticus*, *Microtus epiroticus*, *Crocidura suaveolens* and *Mus macedonicus*, were captured. Two out of 22 *Apodemus sylvaticus* specimens were seropositive for *B. afzelii* by enzyme-linked immunosorbent assay as confirmed by Western blotting, however cultures of skin and bladder samples from all small mammals in Barbour–Stoenner–Kelly's medium-II remained negative for *B. burgdorferi s.l.* All sera tested were negative for *Anaplasma phagocytophilum* by indirect immunofluorescent assay. The prevalence of *B. burgdorferi s.l.* and *Anaplasma phagocytophilum* is low in wild mice of the Asian part of Northern Turkey.

INTRODUCTION

Lyme borreliosis is a prevalent tick-borne zoonosis [1–3] in Europe and the United States, transmitted by ticks, *Ixodes ricinus* and *I. scapularis* respectively. Human granulocytic ehrlichiosis (HGE) is an emerging tick-associated febrile disease which was first described in 1994 based on cases in Wisconsin and Minnesota [4, 5] and caused by infection of *Anaplasma* (*An.*) *phagocytophilum* [6]. The distribution of this illness overlaps with that of *I. ricinus* and *I. scapularis* [7, 8]. Antibodies against *An. phagocytophilum* have been found in patients with Lyme borreliosis and co-infections are common [9, 10].

Peromyscus leucopus (white-footed mice) are natural reservoirs for *Borrelia burgdorferi sensu lato* (*s.l.*) and *An. phagocytophilum* in the United States. Antibodies to *An. phagocytophilum* have been detected in wild mice captured in Connecticut, Minnesota and Rhode Island [11–14]. Additionally, antibodies to all agents may co-exist in white-footed mice [11, 12]. In European countries, small rodents such as *Apodemus*

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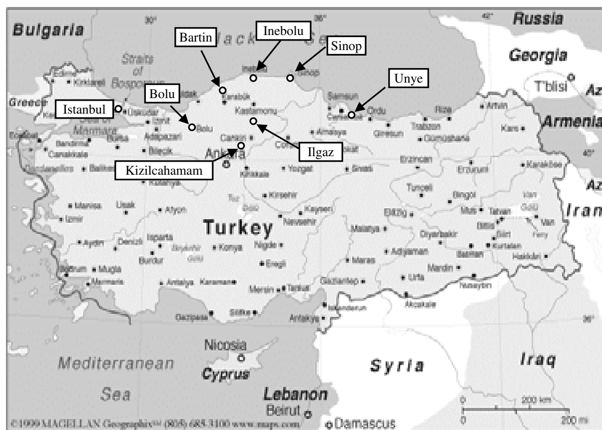

Fig. 1. Site map where small mammals were captured around the Black Sea coast of the Asian side of Turkey.

(*Ap.*) *sylvaticus* (wood mouse), *Ap. flavicollis* (yellow-necked mouse), *Clethrionomys glareolus* (bank vole) are the primary reservoirs of *B. burgdorferi s.l.* [15, 16] and are also implicated as a reservoir of *An. phagocytophilum* [17].

We recently reported the isolation of *B. burgdorferi s.l.* from *I. ricinus* ticks sampled at Trakya, on the European side of the Black Sea coast in Northwestern Turkey [18]. All of the *B. burgdorferi s.l.* genospecies [*B. burgdorferi sensu stricto* (*s.s.*), *B. garinii*, *B. afzelii*, *B. valasiana* and *B. lusitaniae*] known to be carried by *I. ricinus* complex were isolated. The overall infection rate among ticks was 4% and highest rates (16·6%) were found in the Langoz Forest located on the Black Sea coast. Our study is the first attempt to identify rodent species which might be reservoirs for these bacteria in Turkey.

METHODS

Study area

The study area included the national parks of Bolu (Yedigoller National Park), Kizilcahamam (Soguksu National Park), Bartin, Ilgaz (Ilgaz Mountain National Park), Inebolu, Sinop, and Unye in the Black Sea region of Northern Turkey (Fig. 1). Elevation ranged from sea level (Sinop, Inebolu, Unye) to 1900 m (Ilgaz Mountain National Park). Climatic conditions were not remarkable; however, in February, March, June and July, precipitation values were 35% below average; especially in March (27·4%), June (62·9%) and July (13·0%), decreases in the rainfall values were observed compared to the normal precipitation (Archives of the National Meteorological Observatory, Turkey). Ecologically, deciduous forests and mixed forests (evergreen pine and deciduous trees) were surveyed during the expedition.

Isolation of *Borrelia*

We trapped small mammals using Sherman box traps baited with dry food pellets. Traps were set at dusk and collected next morning. Animals were anaesthetized with ether, attached ticks were removed with forceps, placed in vials and saved for further studies. Blood was drawn by cardiac puncture, and serum was separated after centrifugation of the clotted blood samples at 2000 ***g*** for 15 min. Ear tissue samples taken by punch biopsy, and portions of urinary bladders dissected by using sterile forceps and scissors were directly added to Barbour–Stoenner–Kelly's medium-II with 6% rabbit serum [19]. Cultures were incubated at 33 °C for 3 months for *Borrelia* growth.

ELISA

ELISA was performed to detect antibodies against *B. burgdorferi s.l.* in rodent sera by using *B. burgdorferi* strain 297 and *B. afzelii* PGau as antigen [20]. The preparation of antigen and the ELISA were done according to the methods described by Magnarelli et al. [21]. A 1:1000 diluted affinity-purified horseradish peroxidase (HRP)-labelled goat anti-mouse IgG–IgM–IgA serum (Kirkegaard & Perry Laboratory, Gaithersburg, MD, USA) was used as the secondary antibody.

The 1:100 diluted sera from a variety of small mammal species were adsorbed as antigen on 96-well immunoassay plates (Maxisorp Immuno Plates, Nalgen Nunc, Naperville, IL, USA) overnight to confirm the reactivity of HRP-labelled antiserum with immunoglobulin from the wild mammals. After adsorption, HRP-labelled antiserum was reacted and substrate was added to the plates similar to the ELISA procedure. Sera were collected from *Ap. flavicollis*, *Ap. sylvaticus*, *Microtus epiroticus*, *C. suaveolens*, *Mus macedonicus* and normal laboratory ddY mice (SLC, Hamamatsu, Japan). The HRP-labelled anti-mouse IgG–IgM–IgA serum used in this study reacted to sera from mammals classified in the order Rodentia, in contrast to sera from the order Insectivora, such as *C. suaveolens*. Although the widest range of reactivity was observed with the HRP-labelled anti-mouse IgG–IgM–IgA serum, sera

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Table. *Antibodies against* B. burgdorferi sensu lato *and* An. phagocytophilum *in small mammals captured in Northern Turkey between 24 and 31 August 2001*

Location	Species	Number and sex of rodents[a]	Isolation of *Borrelia* in BSK II medium	Antibodies against *B. burgdorferi sensu stricto*	Antibodies against *B. afzelii*	Antibodies against *An. phagocytophilum*
Bolu	*Apodemus flavicollis*	1 (1 M)	0	0	0	0
(Yedigoller National Park)	*Microtus epiroticus*	5 (4 F, 1 M)	0	0	0	0
Kizilcahamam (Soguksu National Park)	*Apodemus flavicollis*	6 (3 F, 3 M)	0	0	0	0
Bartin	*Apodemus flavicollis*	4 (3 F, 1 M)	0	0	0	0
	Crocidura suaveolens	1 (1M)	0	n.t.[b]	n.t.	n.t.
Ilgaz	*Apodemus flavicollis*	2 (1 F, 1 M)	0	0	0	0
(Ilgaz Mountain National Park)	*Apodemus sylvaticus*	7 (2 F, 5 M)	0	0	1	0
	Microtus epiroticus	7 (6 F, 1 M)	0	0	0	0
	Mus macedonicus	1 (1M)	0	0	0	0
Inebolu	*Apodemus sylvaticus*	1 (1 M)	0	0	0	0
Sinop	*Apodemus sylvaticus*	10 (3 F, 7 M)	0	0	0	0
	Crocidura suaveolens	7 (4 F, 3 M)	0	n.t.	n.t.	n.t.
	Mus macedonicus	1 (1F)	0	0	0	0
Unye	*Apodemus sylvaticus*	4 (4M)	0	0	1	0
	Crocidura suaveolens	6 (2 F, 4 M)	0	n.t.	n.t.	n.t.
	Microtus epiroticus	1 (1 F)	0	0	0	0
	Mus macedonicus	1 (1 F)	0	0	0	0
Total		65	0	0	2	0

BSK II medium, Barbour–Stoenner–Kelly's medium-II.
Antibodies reactive to *Borrelia* and *An. phagocytophilum* were detected by ELISA (enzyme-linked immunosorbent assay) and IFA (indirect immunofluorescent assay) respectively.
[a] M, male; F, female.
[b] n.t., Not tested.

from *C. suaveolens* were not tested further in the study. The cut-off value for the ELISA was defined as the mean optical density+2 S.D. of 11 negative control sera from *Ap. agrarius* captured in a rice field in Chunchon, Korea, an area without Lyme borreliosis. Pooled sera from ddY mice infected with *B. afzelii* strain PGau was used as a positive control.

Western blotting

Western blotting was performed as described previously [22]. *Borrelia* cells were electrophoresed in 12·5% polyacrylamide gel and then transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA). The sera diluted with 5% skim milk-PBS were incubated with the membrane for 1 h. After washing, 1:1000 diluted HRP-labelled anti-mouse IgG–IgM–IgA was incubated with the membrane for 1 h. The reactive bands were visualized through incubation with a substrate solution composed of 4-chloro-1-naphtol and hydrogen peroxide.

Indirect immunofluorescent assay (IFA) for the detection of *An. phagocytophilum* antibodies

IFA was performed as described by Unver et al. [23]. Briefly, *An. phagocytophilum* strain HZ propagated in HL-60 cells was applied to each well of a 14-well slide, air dried, and fixed in cold acetone for 5 min. The initial dilution of sera was 1:20 followed by two-fold dilutions. Specific antibody binding was demonstrated by incubating with 1:50 diluted fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG conjugate (Jackson Immuno Research Laboratories Inc., Baltimore, PA, USA) as the secondary antibody. The reactivity of FITC-labelled anti-mouse sera with IgG of wild mammals was examined by the ELISA procedure as described above with some modifications. Binding of FITC-labelled antibody to IgG

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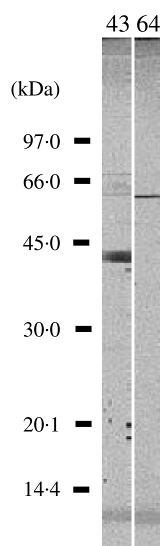

Fig. 2. Demonstration of antibody reactive to *B. afzelii* strain P/Gau by Western blotting. *Borrelia* cells were electrophoresed in 12·5% polyacrylamide gel and then transferred onto PVDF membrane. The 1:100 diluted sera (nos. T43 and T64) showing positive reaction on ELISA were incubated with the membrane. After washing, 1:1000 diluted HRP-labelled anti-mouse IgG–IgM–IgA was incubated and then the reactive bands were visualized by adding a substrate solution.

of mammals was demonstrated by reacting with the HRP-labelled anti-FITC rabbit serum.

RESULTS

During the expedition, 65 small mammals including several mouse species (13 *Microtus epiroticus*, 13 *Ap. flavicollis*, 22 *Ap. sylvaticus*, 14 *C. suaveolens*, and 3 *Mus macedonicus*) were captured. *Apodemus* spp. accounted for approximately 50% of all collected animals followed by insectivore *C. suaveolens* species (see Table). The highest number of rodents were captured in Ilgaz Mountain National Park and in Sinop. Two sera (nos. T43 and T64) of *Ap. sylvaticus* specimens captured in Sinop and Unye respectively, showed significant reactivity against *B. afzelii* by using ELISA technique; an optical density of more than 2·0 (the mean optical density+2 s.d. of 11 negative control sera from *Ap. agrarius* captured in a rice field in Chunchon, Korea, an area without Lyme borreliosis), in contrast to *B. burgdorferi* strain 297 (see Table). Both sera showed several reactive bands and the seroreactivity was confirmed by Western blotting using *B. afzelii* as antigen (Fig. 2).

The urinary bladder and ear-punch biopsy samples of small mammals remained negative for *B. burgdorferi s.l.* agent after 3 months incubation at 33 °C. Furthermore, none of the rodents that we captured had *An. phagocytophilum* antibodies as determined by IFA. Some of the rodents (*Apodemus* spp. and *C. suaveolens*) were infested with ixodid tick larvae, however, they were culture-negative for *B. burgdorferi s.l.*

DISCUSSION

The expedition was conducted in the summer of 2001 on the Asian side of the Black Sea region of Turkey where some Lyme borreliosis cases have been reported [24–27]. In general, climatic conditions, rainfall values and ecological features constitute a suitable habitat for vectors and reservoir animals of *B. burgdorferi s.l.* on the Black Sea coast on both the Asian and European sides of Northern Turkey.

We recently reported the first isolation of *B. burgdorferi s.l.* from *I. ricinus* ticks sampled at Trakya, the European part of the Black Sea coast of Northwestern Turkey [18]. All of the *B. burgdorferi s.l.* genospecies (*B. burgdorferi s.s.*, *B. garinii*, *B. afzelii*, *B. valasiana*, and *B. lusitaniae*) known to be carried by *I. ricinus* were isolated. Overall infection rate among ticks was 4% and the highest values (10%) were found in the Langoz Forest located at the Black Sea coast. We expected to find a high infection rate of *B. burgdorferi s.l.* among rodents and attached ticks sampled from the Asian side of the Black Sea region, but it was very low (3·1%) as determined by ELISA. We attempted to isolate *B. burgdorferi s.l.* from urinary bladder and ear-punch biopsy material, a method proven reliable for the isolation of this bacterium [28, 29]. However, we could not isolate spirochetes from small mammals including seropositive animals. These seropositive mice might have antibodies retained from recent *Borrelia* infections. *Apodemus*, *Clethrionomys*, *Microtus* and *Mus* spp. were proven to be reservoirs of *B. burgdorferi s.l.* agent in Europe [16]. Wood mouse (*Ap. sylvaticus*) and yellow-necked mice (*Ap. flavicollis*) have been reported as reservoirs of *B. burgdorferi s.s.* and *B. afzelii*. Therefore, the rodent species that we captured were proven reservoirs of *B. burgdorferi s.l.* genospecies. However, we could not isolate *Borrelia* from these animals. The low infection rates among mice might be due to the low vector activity which might result from the unusually low precipitation during

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the summer of 2001 compared to the average annual rainfall values of the last 50 years (35% below normal in March, June and July of 2001). In this region, normal precipitation is 32·6 mm in June, however only 12·1 mm was observed (62·9% less than average) in 2001. Dry conditions may influence the distribution and life-cycle of vector ticks and reservoir hosts of *B. burgdorferi s.l.* During the survey, we did not capture any free-living ticks on plant surfaces and ground leaf cover with white flannel flags.

Similar to *Borrelia*, *An. phagocytophilum* have been implicated to reside in small mammals, particularly *Ap. sylvaticus* and *Ap. flavicollis* in Europe [17]. In this study, none of the small mammals had antibodies against *An. phagocytophilum*. This may suggest that the transient dry period in the Asian part of Northern Turkey during the summer of 2001 may have resulted in a low infection rate with *B. burgdorferi s.l.* and *An. phagocytophilum* among small wild mammals.

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