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High Prevalence of Granulocytic Ehrlichiae and *Borrelia burgdorferi* Sensu Lato in *Ixodes ricinus* Ticks from Bulgaria

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Bulgarian *Ixodes ricinus* ticks were examined for *Ehrlichia* and *Borrelia* coinfection: 34 and 32% of adult ticks and at least 2 and 10% of nymphs were positive for these infections, respectively. Coinfections and dual or triple *Borrelia* infections were frequent, although *Ehrlichia phagocytophila* heterogeneity was minimal. Multiple tick-borne bacteria coexist in *I. ricinus* ticks in southeastern Europe.

Lyme borreliosis and human granulocytic ehrlichiosis (HGE) are emerging infections sometimes cotransmitted by *Ixodes* species ticks, including *Ixodes ricinus* in Europe (3, 4, 14, 16, 17, 19, 25). Lyme borreliosis is the most common vector-borne disease in the Northern Hemisphere, but HGE is still poorly investigated and its geographic range is unclear. Five different species of *Borrelia burgdorferi* sensu lato are described in Europe—*B. burgdorferi* sensu stricto, *Borrelia garinii, Borrelia afzelii, Borrelia valaisiana*, and *Borrelia lusitaniae* (2, 5, 11–13, 20, 28). However, the causative agent of HGE is a granulocytic ehrlichia conspecific with the veterinary pathogens *Ehrlichia phagocytophila* and *Ehrlichia equi*.

Lyme borreliosis is endemic in Bulgaria (7). Despite the lack of mandatory Lyme borreliosis reporting in Bulgaria, about 500 cases are reported annually (4 cases/100,000 persons). Similarly, 9% of Bulgarian patients with tick bites have antibodies to the HGE agent (8), suggesting that the HGE agent is probably frequent in tick populations. In the present study, Bulgarian ticks were tested for *B. burgdorferi* and the HGE agent, and the findings reveal a higher prevalence of infection and coinfection than previously reported in Europe.

In the summer of 2000, *I. ricinus* ticks were collected by flagging vegetation in wooded areas near Sofia, Bulgaria. After identification, ticks were processed individually if adults and in pools of five if nymphs. Each tick or pool was mechanically homogenized in 10 mM Tris, 1 mM EDTA, 100 μg of proteinase K per ml, and 0.5% sodium dodecyl sulfate lysing buffer, incubated at 60°C for 1 h, boiled for 10 min, and treated with 5 M NaCl and hexadecyltrimethylammonium bromide at 65°C for 20 min, followed by DNA extraction in phenol-chloroform. DNA was precipitated with isopropanol, washed with ethanol, dried, and dissolved in 30 μl of 10 mM Tris (pH 8).

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For amplification of *Ehrlichia* DNA, the *ankA* gene PCR, described by Walls et al. (27), was performed. Each PCR run included a known positive control (*E. equi*-infected horse neutrophil DNA) and a negative control (water blank). For detection of *B. burgdorferi* sensu lato DNA, a similar PCR amplification method was used with the *fla* gene primers BBSCH31 and BBSCH2 (23). To confirm *ankA* PCR amplification, the 16S rRNA gene primers ge9f and ge10r were used in a PCR conducted in a separate laboratory (6). For specific detection of *B. burgdorferi* sensu lato amplicons, the reverse line blotting technique was performed as previously described (24).

Six 16S rRNA HGE agent PCR products were cloned into the pCR4-TOPO (Invitrogen, Inc., San Diego, Calif.) vector and sequenced. Sequences were compared with 16S rRNA sequences of the HGE agent from Wisconsin (GenBank accession number U02521). Alignments of 16S rRNA gene sequences in ticks with other *Ehrlichia* sequences were conducted using ClustalX (version 3.5c) and were used to generate distances and dendrograms.

Unfed *I. ricinus* ticks (202) were examined for the presence of the *E. phagocytophila* genogroup and *B. burgdorferi* sensu lato DNA. The sex and stage distribution of ticks by infection are given in Table 1. Of 112 adult ticks, 38 (34%) and 36 (32%) contained the *E. phagocytophila* genogroup and *B. burgdorferi* sensu lato DNA, respectively, and 15 ticks were coinfected. Of 18 nymph pools, 9 were infected with *B. burgdorferi* sensu lato and 2 were coinfected. Of the 17 coinfected samples, 16 were confirmed with reverse line blot assay (Table 2). The reverse line blotting also detected *Ehrlichia* DNA in 5 of 20 *B. burgdorferi*-only PCR-positive samples, suggesting an even higher proportion of coinfected ticks (Table 2). Overall, *B. afzelii* was detected in 19 (17%) of the 112 adult ticks (Table 2). Dual infections with *Borrelia* species were noted often.

Only 19 of 50 *ankA*-positive samples were also amplified with the 16S rRNA gene primers. A higher percentage of coinfected samples (73.3%, 11 of 15) was detected by reverse line blot assay than by the 16S rRNA gene PCR. However, all

TABLE 1. Results of *ankA* and *fla* PCR amplification for detection of the *E. phagocytophila* genogroup and *B. burgdorferi* sensu lato DNA in *I. ricinus* ticks

Stage and sex	No. of ticks	No. (%) of ticks infected with:		
		E. phagocytophila genogroup	*B. burgdorferi* sensu lato	*E. phagocytophila* and *B. burgdorferi*
Adult				
Female	62	19 (30.6)	20 (32.3)	8 (12.9)
Male	50	19 (38)	16 (32)	7 (14)
Total	112	38 (33.9)	36 (32.1)	15 (13.4)
Nymph	90[a]	2 (2.2)	9 (10)	2 (2.2)
Total	202	40 (19.8)	45 (22.3)	17 (8.4)

[a] Nymphs were processed in 18 pools of five ticks each.

six sequenced PCR amplicons from ticks were identified as *E. phagocytophila* group (99.8 to 99.9% identity with the HGE agent [GenBank accession number U02521]) (1).

This study demonstrates coinfection of granulocytic ehrlichiae and *B. burgdorferi* sensu lato and heterogeneity of *B. burgdorferi* sensu lato in unfed *I. ricinus* ticks in Eastern Europe. Although *E. phagocytophila* group species have been frequently detected in *I. ricinus* ticks in Europe (3, 9, 14, 15, 18, 22, 24, 26), the 33.9% *E. phagocytophila* group tick infectivity rate in this study is high and potentially explains the rate of HGE seropositivity with undifferentiated febrile illnesses after tick bites in Bulgaria (8). Based on minimal 16S rRNA gene sequences, heterogeneity of the ehrlichiae appears minimal. The 13% prevalence of coinfection with *B. burgdorferi* sensu lato in adult ticks from Bulgaria is higher than reported previously in Europe and is similar to the 1.9 to 29.6% rates demonstrated in the United States (10, 11, 21, 22). The prevalence of coinfected ticks supports findings where 9.7% of Bulgarian patients with early Lyme borreliosis had serological evidence of HGE (8).

The adult ticks in this study demonstrated a prevalence of *B. burgdorferi* sensu lato similar to that of granulocytic ehrlichiae. Nymphs had a minimal infection rate of 10%, which is intermediate in prevalence compared with European rates that vary from 2 to 43% for nymphs and from 3 to 58% for adults. *B. afzelii* was the predominant (17%) genospecies detected in the Bulgarian adult ticks. A few ticks were infected with each of the other *B. burgdorferi* sensu lato, and dual *Borrelia* infections were found, including two cases of infection with *B. valaisiana* and *B. afzelii* and cases of infection with *B. lusitaniae* and *B. garinii* in one adult and two nymphal pools. One tick was infected with a *B. afzelii*-like species recently detected in ticks from St. Petersburg, Russia (1).

The results show that a high proportion of ticks infected with the *E. phagocytophila* genogroup and *B. burgdorferi* sensu lato are present in Bulgaria and southeastern Europe. These findings in Bulgarian ticks should alert southeastern Europe to the possibility of human infections. Moreover, since *I. ricinus* is frequently infected with both pathogens, simultaneous HGE and Lyme borreliosis is probably not uncommon. Thus, every case of Lyme borreliosis with atypical clinical manifestations (29) should be carefully examined for the possibility of concurrent HGE.

TABLE 2. Results of the reverse line blot assay of 17 DNA samples positive by both the *Ehrlichia* (*ankA*) and *Borrelia* (*fla*) PCR and 20 samples positive by the *Borrelia* (*fla*) PCR but negative by the *Ehrlichia* (*ankA*) PCR

PCR result	Sex[a] or stage	No. of ticks infected with:														
		HGE agent or *E. phagocytophila*	Genus *Ehrlichia*	Total *Ehrlichia*	*B. burgdorferi* sensu stricto	*B. garinii* or *B. afzelii*-like	*B. afzelii*	*B. valaisiana*	*B. valaisiana*, or *B. burgdorferi* sensu stricto, and *B. afzelii*	*B. lusitaniae*, *B. burgdorferi* sensu stricto, and *B. garinii*	*B. lusitaniae* and *B. garinii*	*B. burgdorferi* sensu lato	*B. burgdorferi* sensu stricto and *B. afzelii*	*B. garinii* and *B. afzelii*	Total *Borrelia*
Positive for *Borrelia* and *Ehrlichia*	Female	6		6	1		5				1		1		8
	Male	7		7	1		3					1	1		6
	Nymph	2		2		1	1								2
Total		15		15	2	1	9				1	1	2		16
Positive for *Borrelia* and negative for *Ehrlichia*	Female	2	2	4			5	1						1	7
	Male	2	2	4	1		1		1						3
	Nymph	1	1	2		1	2			1	1			1	6
Total		5	5	10	1	1	8	1	1	1	1			2	16

[a] Sex identified for adult ticks.

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