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Short communication

Northern white-breasted hedgehogs *Erinaceus roumanicus* as hosts for ticks infected with *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in Romania

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ABSTRACT

Borrelia burgdorferi sensu lato and *Anaplasma phagocytophilum* are two tick-borne pathogens of medical and/or veterinary importance which are distributed worldwide. *Erinaceus roumanicus*, the Northern white-breasted hedgehog, is a common synanthropic species that is known to carry not only the hedgehog tick, *Ixodes hexagonus*, but also *I. ricinus*, the most common European tick species. *I. ricinus* is the main vector of both mentioned pathogens. Within this framework and because only limited information is available on the role of *E. roumanicus* in the ecology of *B. burgdorferi* s.l. and *Anaplasma phagocytophilum* in Europe, we carried out an epidemiological surveillance on this species in Romania. From the 57 examined hedgehogs collected in 12 different counties, 24 presented tick infestation. Most ticks ($n = 959$) were morphologically identified as larvae, nymphs, or adults of *I. ricinus*. The prevalence of *B. burgdorferi* s.l. was 0.4%, and that of *A. phagocytophilum* 12%. In all positive cases for *B. burgdorferi* s.l., restriction fragment length polymorphism revealed the genospecies *B. afzelii*. In Romania, only limited information is available on the epidemiology of *B. burgdorferi* s.l. and *A. phagocytophilum*. As hedgehogs commonly share the same environment with humans and other potential reservoir hosts for tick-borne pathogens, our study provides new epidemiological data of public health importance.

Introduction

Hedgehogs are common nocturnal insectivores (Silaghi et al., 2012) living in rural, suburban, and urban habitats (Marié et al., 2012). Due to their active foraging behavior in undergrowth, they are ideal hosts for ticks (Földvári et al., 2012). One of the most common tick species on hedgehogs is *Ixodes ricinus*, an exophilic, questing tick, which has also been frequently reported on humans (Briciu et al., 2011; Gern et al., 1997). *I. ricinus* is a known vector of both *Borrelia burgdorferi* sensu lato (s.l.) and *Anaplasma phagocytophilum*. Both European hedgehog species and their ticks have been shown to host different *B. burgdorferi* s.l. genospecies (Skuballa et al., 2007, 2012) as well as other tick-borne pathogens, such as *A. phagocytophilum* (Skuballa et al., 2010).

B. burgdorferi s.l. is the causative agent of Lyme borreliosis, one of the most common tick-borne diseases in humans and animals in Europe and the USA (Skuballa et al., 2012). *A. phagocytophilum*, an emerging pathogen of public health importance (Jiang et al., 2011), is an obligate intracellular bacterium, occurring worldwide in animals and humans (Skuballa et al., 2010).

The maintenance of tick-borne pathogens in nature follows a variety of patterns, involving ticks and reservoirs hosts. *Erinaceus roumanicus*, the Northern white-breasted hedgehog, inhabits central and eastern Europe (Dziemian et al., 2010). Based on the large diversity of ectoparasites they harbor and the high prevalence of infestations recorded as well as their anthropophilic behavior, hedgehogs can play an important role in the epidemiology of tick-borne pathogens (Földvári et al., 2012).

Most published data concerning ixodid ticks feeding on hedgehogs and their role in the complex cycle of tick-borne pathogens refers to *E. europaeus*, with only limited information on

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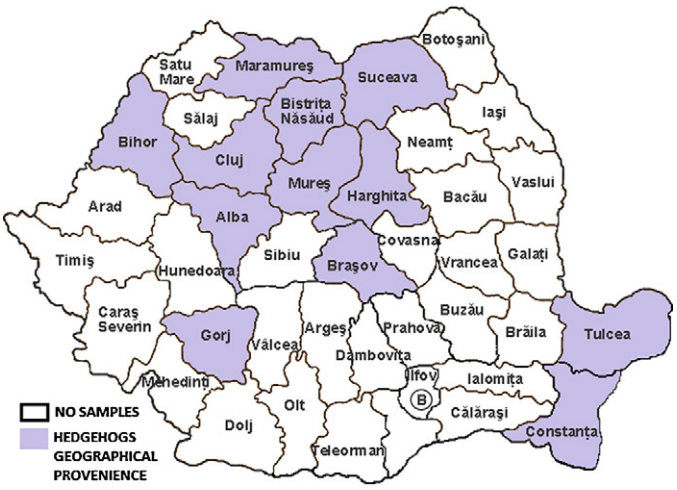

Fig. 1. Sampling sites of the hedgehogs.

E. roumanicus being available despite its widespread distribution (Dziemian et al., 2010).

The aims of this study were to investigate tick populations parasitizing *E. roumanicus* and to provide epidemiological data regarding their association with the 2 tick-borne pathogens of public health importance: *B. burgdorferi* s.l. and *A. phagocytophilum*.

Materials and methods

Hedgehogs and ticks

In April and May of 2011 and 2012, 57 free-roaming living hedgehogs from 12 different counties in Romania (Fig. 1) were brought to the laboratory of the Department of Parasitology and Parasitic Diseases, Cluj-Napoca. For a proper examination, each hedgehog was anesthetized using the inhalation technique, which is preferred in all insectivores (West et al., 2007). The anesthesia was induced with isoflurane 5% in 100% oxygen administered into an induction chamber connected to a non-rebreathing system. The anesthesia was also maintained with isoflurane 2% in oxygen delivered through a mask held over the animal's nose (Mullineaux et al., 2003). After examination, each hedgehog was kept under surveillance until full recovery for 24 h and released back to the capture area.

All parasitizing ticks were collected, regardless of species and life stage, and stored in pure ethanol. Species-specific identification was performed using the morphological keys in Feider (1965) under a binocular microscope.

DNA extraction

Genomic DNA extraction was performed individually on all ticks. DNA extraction was carried out using QIAGEN DNeasy Blood & Tissue Kit (Protocol: Purification of Total DNA from Animal Tissues).

Polymerase chain reaction (PCR)

PCR to detect *B. burgdorferi* s.l. and *A. phagocytophilum* DNA was performed on all samples.

The PCR for detecting *B. burgdorferi* s.l. was carried out by the method described by Priem et al. (1997), and the amplification was performed targeting the *ospA* gene (Priem et al., 1997), using forward primer OspAOut 1 (5′-GGGAATAGGTCTAATATTAGCC-3′) and reverse primer OspAOut 2 (5′-CACTAATTGTTAAAGTGGAAGT-3′). Each time the PCR was performed, positive control samples were included (strains of *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*), and laboratory-reared uninfected *I. ricinus* ticks were used as negative controls. The genomic DNA was extracted with the same general method. The primer pair used for detecting *A. phagocytophilum* was: forward primer (5′-ATGGAAGGTAGTGTTGGTTATGGTATT-3′ and reverse primer 5′-TTGGTCTTGAAGCGCTCGTA-3′), targeting *msp2* gene (Courtney et al., 2004).

Nested PCR

For samples PCR-positive for *B. burgdorferi* s.l., nested PCR was performed. A total of 4 μl of first-round PCR product was employed for the nested-PCR with OspAIN 1 (5′-GCAAAATGTTAGCAGCCTTGAT-3′) as the forward primer and OspAIN 2 (5′-CTGTGTATTCAAGTCTGGTTCC-3′) as the reverse primer. The amplification profile consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing 42 °C for 30 s, and extension at 72 °C for 30 s.

The amplification was performed in a BIO RAD C1000™ Thermal Cycler. Nested-PCR was performed including negative and positive control samples. Each time, aliquots of the PCR product were electrophoresed on 1.5% agarose gel and stained with SYBR® Safe DNA gel stain (Invitrogen) and observed for the presence of the specific fragment under UV light (BIO DOC-ItTM Imagine System). DNA fragment size was compared with a standard molecular weight (100 bp DNA ladder, Fermentas).

Restriction fragment length polymorphism (RFLP)

Ten microliters of aliquots of nested PCR amplification products were subjected to RFLP analysis by digestion carried out according to the manufacturer's instructions with 10 μg/μl of restriction enzymes MseI (Fermentas) and 2 μg/μl of AlwI (Fermentas). The digested fragments were visualized in 4% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen) (Menardi et al., 2008; Floris et al., 2007).

Statistical analysis

Frequency (number of positive samples), prevalence (percent of positive samples from total examined), and the 95% confidence interval (CI) were calculated for ticks on hedgehogs as well as for the infection with *B. burgdorferi* s.l. and *A. phagocytophilum* in ticks. All these parameters were determined for each life stage of ticks (larva, nymph, and adult – female and male). The difference in prevalence between groups was statistically analyzed by chi-squared independence test. A p value of <0.05 was considered statistically significant. All statistics were performed using the EpiInfo 2000 software.

Results

Of the 57 hedgehogs examined, 24 had a tick infestation. Most ticks (altogether $n = 959$) were *I. ricinus.* Two *Rhipicephalus* sp. from Tulea county and one *Dermacentor* sp. from Cluj county were found on 2 hedgehogs. However, these ticks were excluded from further analysis due to their low prevalence. The prevalence based on life stage was as follows: larvae 16.5% (158/959; 95% CI: 14.2–19.0%), nymphs 55.3% (530/959; 95% CI: 52.1–58.4%), females 21.8% (209/959; 95% CI: 19.2–24.6%), and males 6.5% (62/959; 95% CI: 5.0–8.3%) (Table 1).

The overall prevalence of *B. burgdorferi* s.l. in the ticks was 0.4% (4/959; 95% CI: 0.1–1.1%). The positive results were obtained for 3 nymphs and 1 male. From all 24 hedgehogs that were infested with *I. ricinus*, 2 presented *B. burgdorferi* s.l.-positive ticks (8.3%;

Table 1
Prevalence of *B. burgdorferi* s.l. and *A. phagocytophilum* DNA in ticks collected from hedgehogs.

Tick life stage	No. of ticks	Frequency	Prevalence (%)	95% CI	*p*
B. burgdorferi s.l.					
Larva	158	0	0	0.0–2.3	0.2264
Nymph	530	3	0.6	0.1–1.8	
Female	209	0	0	0.0–1.7	
Male	62	1	1.6	0.0–8.7	
Total	959	4	0.4	0.1–1.1	
A. phagocytophilum					
Larva	158	4	2.5	0.7–6.4	0.00001
Nymph	530	101	19.1	15.9–22.7	
Female	209	7	3.3	1.4–6.8	
Male	62	3	4.8	1.0–13.5	
Total	959	115	12	10.0–14.3	

2/24; 95% CI: 1.1–28.0) (Table 1). In all cases, the genospecies was identified as *B. afzelii*.

A total of 115 out of 959 ticks (12%; 95% CI: 10.0–14.3%) was positive for *A. phagocytophilum* (Table 1). Of these, 101 were nymphs. Eight hedgehogs that presented *I. ricinus* infestation had *A. phagocytophilum*-positive ticks (34.8%; 8/23; 95% CI: 16.4–57.3). The highest prevalence of *A. phagocytophilum* DNA was present in nymphs (19.1%; 101/530; 95% CI: 15.9–22.7).

Two hedgehogs (8.3%; 2/24; 95% CI: 1.1–28.0) had ticks positive for *B. burgdorferi* s.l. and *A. phagocytophilum*.

One tick was positive for both investigated pathogens 0.1% (1/959; CI: 0.0–0.7%).

Discussion

This study presents the results of an epidemiological investigation of 2 tick-borne pathogens, *Borrelia burgdorferi* s.l. and *Anaplasma phagocytophilum* in ticks collected from *Erinaceus roumanicus* in Romania. We identified 3 genera of hard ticks parasitizing this hedgehog species: *Ixodes*, *Dermacentor*, and *Rhipicephalus*. All of the *Ixodes* ticks were identified as *I. ricinus*. In a recent study on host–tick associations in Romania, Mihalca et al. (2012) found 6 tick species on hedgehogs: *I. ricinus*, *Haemaphysalis punctata*, *Dermacentor marginatus*, *Rhipicephalus sanguineus* s.l., *R. rossicus*, and *Hyalomma marginatum*. *I. hexagonus*, also called the hedgehog tick, has only been rarely reported in Romania (Feider, 1965) and has not been associated with *E. roumanicus* in this country recently (Mihalca et al., 2012).

The high level of tick infestation in hedgehogs (42.1%) and the predominance of *I. ricinus* could be correlated with the seasonal dynamics of this tick species in Romania, where a spring density peak in April and May occurs (Coipan et al., 2011).

A. phagocytophilum is well established as a tick-borne agent of veterinary importance worldwide, and it is considered to be an emerging human pathogen (Santos et al., 2004). The ecology of *A. phagocytophilum* is complex and still not well defined, but it is believed to be maintained in nature in a tick–vertebrate cycle with the involvement of *Ixodes* spp., similarly to that of *B. burgdorferi* s.l. (Skuballa et al., 2010; Santos et al., 2004). It is known that *I. ricinus* ticks acquire *A. phagocytophilum* when taking a blood meal from an infected animal and can transfer the pathogen during the next blood meal (i.e. on another host) (Polin et al., 2004). The overall prevalence of *A. phagocytophilum* in ticks collected from hedgehogs in our study was 12%. Skuballa et al. (2010), in a similar study, showed that this pathogen has a prevalence of 25.8% in organ pools and 39.5% in engorged female ticks collected from an experimental hedgehog population in Germany. The difference between the results could be explained by the reduced number of analyzed samples, 38 engorged samples, and by the fact that only adult female ticks were tested. Analyzing samples as pools could also influence the results. Another explanation could be possible differences between *E. roumanicus* and *E. europaeus* ecology.

The role of hedgehogs as hosts of *B. burgdorferi* s.l. is of considerable epidemiological interest. The results of a study from Germany showed that hedgehogs may harbor at least 3 *B. burgdorferi* genospecies, all of which are known (*B. afzelii*, *B. garinii*) or are strongly suspected (*B. spielmanii*) of being pathogenic for humans (Skuballa et al., 2007). All our positive samples with *B. burgdorferi* s.l. were identified as *B. afzelii*. In another study on the occurrence of different *B. burgdorferi* s.l. genospecies in hedgehogs and their ticks, infection was detected in the tissues of 35/227 *E. europaeus* and 2/10 *E. roumanicus* (Skuballa et al., 2012). In *E. europaeus*, the authors detected 3 genospecies: *B. afzelii*, *B. bavariensis*, and *B. spielmanii* and in *E. roumanicus*, *B. afzelii* and *B. bavariensis*; *B. afzelii* being the dominant genospecies, an aspect that was also revealed by our research. Of the *I. ricinus* ticks, 4 out of 45 females were infected (8.9%), once again *B. afzelii* was present in 3 of them and *B. spielmanii* in the other (Skuballa et al., 2012). Gern et al. (1997) investigated the reservoir role of hedgehogs for *B. burgdorferi* s.l. Thirteen animals were examined from which they collected *I. ricinus* and *I. hexagonus*. All but one hedgehog harbored *B. burgdorferi* s.l.-infected ticks, with an infection rate of 27% (97/356) and 24% (62/257) for *I. ricinus* and *I. hexagonus* ticks, respectively.

The low prevalence of *B. burgdorferi* s.l. in *Ixodes* ticks removed from hedgehogs in this study (0.4%), could be explained by the fact that a large number of the ticks (nymphs, $n = 140$) were collected from a single hedgehog. All of these individuals were negative.

To our knowledge, this is the first study that evaluates the prevalence of *A. phagocytophilum* and *B. burgdorferi* s.l. in ticks collected from *E. roumanicus* in Romania. The results obtained in this study confirmed the presence of both investigated pathogens, providing tangible eco-epidemiological data. It also emphasizes the necessity of a surveillance of hedgehogs in the context of human tick-borne disease risk.

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