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Original article

Ticks and tick-borne pathogens in South Bohemia (Czech Republic) – Spatial variability in *Ixodes ricinus* abundance, *Borrelia burgdorferi* and tick-borne encephalitis virus prevalence

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ABSTRACT

Spatial distribution of *Ixodes ricinus* tick host-seeking activity, as well as prevalence of *Borrelia burgdorferi* sensu lato and tick-borne encephalitis virus (TBEV) were studied in the TBE endemic area of South Bohemia (Czech Republic). High variability in tick abundance detected in a network of 30 study sites was most closely associated with characteristics of vegetation cover. Of 11,182 tested tick samples, 12% carried DNA of spirochete from *B. burgdorferi* s.l. complex. *B. afzelii* and *B. garinii* prevailed among spirochete species. The presence of *B. spielmanii* in the region was confirmed. The median number of borrelial genome copies in positive samples reached 6.6×10^3 by real-time PCR. The total prevalence of TBEV in pooled samples reached 0.32% (20,057 samples tested), at least one TBEV positive tick was present in 21 out of 30 sampling sites.

Introduction

Concerning the distribution of human tick-borne diseases, the hard tick *Ixodes ricinus* is the most important recognized vector of tick borne pathogens in (Central) Europe. This tick species inhabits an extensive area reaching from Ireland to western parts of Russia and from Scandinavia to North Africa. It is the major vector of the causative agents of Lyme borreliosis (spirochetes of the *Borrelia burgdorferi* sensu lato complex), tick-borne encephalitis (tick-borne encephalitis virus), human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), babesiosis (*Babesia divergens*, *B. microti*), some other less frequent human diseases and pathogens of veterinary importance (Charrel et al., 2004; Parola and Raoult, 2001). With an annual number of over 85 thousand cases of Lyme borreliosis and almost 2900 cases of tick-borne encephalitis (TBE) (ECDC Meeting report, 2012) these two diseases are the vector-borne diseases with largest impact on human health in Europe.

In the last decades ticks and tick-borne pathogens have received increasing attention, both from experts (medical, scientific) and general public. Concerning eco-epidemiological studies the research interest has focused mainly on estimation of tick activity, prevalence of tick-borne pathogens (including meta-analysis) and subsequently, efforts to identify the key factors, that determine the above mentioned parameters and might be used for disease risk prediction (e.g. Hubalek et al., 2003; Medlock et al., 2013; Schwarz et al., 2009, 2012; Swei et al., 2011). In Europe, such studies focus mainly on variability in time, following few local tick populations for several years. Apart from EDEN and EDENext projects, which cover almost whole Europe, studies that take into consideration multiple sampling sites and the spatial variability in tick and tick-borne pathogen abundance are scarce in Central Europe (Altobelli et al., 2008; James et al., 2013; Nazzi et al., 2010; Rizzoli et al., 2002).

To fill the gaps in this field we collected the data in a typical central European tick-borne disease endemic area – the region of South Bohemia in the Czech Republic. An annual average of 23.3 TBE disease cases per 100,000 inhabitants is registered in this region

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compared to 6.5 cases per 100,000 inh. for the whole country (Kriz and Benes, 2011). The first part of our project consisted mainly of extensive collection of data on variability in *I. ricinus* activity, and on distribution of the two major wide-spread tick-borne pathogens: *B. burgdorferi* s.l. and tick borne-encephalitis virus (TBEV). The goal of the second part of our project was accumulation of a large quantity of environmental data, analysis and elucidation of the relationship between environmental variables and spatial distribution of ticks and tick-transmitted pathogens.

Methods

Area under survey

The geographic area of interest is located in the southern part of the Czech Republic – region of South Bohemia. It is well known for a high occurrence of tick-borne diseases. The presence of natural and cultural attractions and a developed tourism industry makes it also an attractive site for high number of visiting tourists. This combination increases the epidemiological impact of the area on public health of local human population as well as visitors. An average of 154 human cases of Lyme borreliosis and 146 human cases of TBE is registered per year in South Bohemia (data provided by the National Institute of Public Health, Prague).

Geographically, climatically and biologically, the area is considerably heterogeneous ranging from 350 m above the sea level (a.s.l.) of the Orlik dam to the highest sections of the Sumava forest mountain range (1378 m a.s.l.). The total area under survey comprised 10,056 km^2. More than 38% of the area is covered by forests. Lower parts are covered mostly by deciduous and mixed forests, whereas in upper parts coniferous forests predominate.

Tick sampling

I. ricinus ticks were collected and their host-seeking activity was estimated by flagging. Based on a thorough GIS analysis of the vegetation cover, altitude, TBE incidence and tourist activity, the areas principally suitable for tick survival (and having epidemiological importance at the same time) were identified (Svec et al., 2009). Subsequently, semi-random selection procedure was run to select 35 potential sites of which later 30 definite study sites were selected in field survey (Fig. 1). GPS coordinates, altitude (ArcPad, ESRI) and basic phytocenological characteristics were recorded in field.

Ticks were collected in three sampling events: April, June/July and September 2008. In each campaign all 30 localities were sampled for a maximum time span of 10 days, each locality for three flaghours (one flaghour defined as one hour of continual flagging of one worker). The numbers of nymphal, male and female adult ticks were recorded. Different development stages, female and male ticks were stored in separate tubes. Tick density (number of individuals per 100 m^2 area) has a close and linear relationship to relative abundance (number of ticks per hour). According to experience obtained in Central European types of habitat 1 flag-hour corresponds approximately to 200 m^2 area (see Danielova et al., 2010). Tick relative abundance was recalculated for tick density (number of ticks per 100 m^2) ensuring comparability with other studies.

Tick samples were transported within hours after flagging in a cooled box to the laboratory where they were stored at −74 °C until isolation of nucleic acids. DNA was isolated from individual ticks, RNA from pooled samples. Using stainless steel beads the ticks were homogenized individually in 200 μl of sterile PBS (phosphate buffered saline, pH 7.4) for 2 min at 30 shakes/s (Tissue Lyser II, Qiagen). After brief centrifugation, 15 μl of each sample was transferred to a pooled sample (maximum of 10 original samples to one pool). The original samples were subjected to DNA isolation, the pooled samples to RNA isolation. DNA and RNA in parallel were extracted from all adult ticks and maximum of 125 nymphal ticks per sampling event and locality. The remaining nymphal samples were directly homogenized as pools of 10 individuals in 400 μl of sterile PBS and only RNA was isolated. Altogether 20,057 ticks were subjected to TBEV RNA detection (in pools) and 11,182 individual ticks were tested for the presence of *B. burgdorferi* DNA.

Detection of tick-borne pathogens

DNA was extracted using a modified Chelex protocol (Rauter et al., 2002; Walsh et al., 1991). Briefly: 350 μl of 10% Chelex® 100 Resin solution in TE (Tris–EDTA) buffer was added to each sample and incubated overnight at 56 °C with shaking. After vortexing the samples were incubated for 10 min at 95 °C and immediately cooled on ice. The samples were centrifuged, the supernatant was collected and either directly used for PCR or stored at −20 °C. RNA was isolated using QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instructions. RNA was used immediately for TBEV detection or stored at −74 °C.

The presence of borrelial DNA was detected by PCR targeting a fragment of o*spA* gene. PCR reactions of 25 μl total volume contained: 10.5 μl of sample and 12.5 μl of Combi PPP Mastermix (Top-Bio) (final concentrations: 75 mM Tris–HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dTTP, 200 μM dGTP, 2.5 U Taq purple DNA polymerase) and 1 μl of each of 10 mM primers SL1 (5′-AATAGGTCTAATAATAGCCTTAATAGC-3′), SL2 (5′-CTAGTGTTTTGCCATCTTCTTTGAAAA-3′) (Demaerschalck et al., 1995). After initial denaturation at 95 °C, the template was amplified in 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s with final elongation step at 72 °C for 3 min. Positive and negative controls were included in each run. PCR products were analyzed by gel electrophoresis. Positive samples were subjected to genospecies identification by reverse line blotting (RLB). *Borrelia* genospecies were identified according to the protocol of Gern et al. (2010). Briefly: a fragment of borrelial 23S–5S intergenic spacer region was amplified by PCR using a biotin-labelled primer and hybridized with multiple species-specific probes (Gern et al., 2010) immobilized on a membrane. After washing, the hybridized products were visualized by chemiluminiscence.

TBEV was detected by two-step RT-PCR amplification of a fragment of gene encoding viral E protein. First, 2 μl of 10 mM reverse primer (E(R): 5′-CCGTTGGAAGGTGTTCCACT-3′) (Ruzek et al., 2007) and 4 μl of template RNA were incubated at 70 °C for 10 min. Subsequently 1.25 μl of 10 mM dNTPs, 20 U of SUPERase-In RNA inhibitor (Ambion), 200 U of M-MLV reverse transcriptase (Promega) and DEPC-treated water to the final reaction volume of 25 μl were added. The cDNA synthesis was conducted at 37 °C for 60 min. Subsequently, 4 μl of cDNA were used for PCR amplification with primers E(F) (5′-GGGGACYACGAGGGTYACCT-3′) and E(R): (5′-CCGTTGGAAGGTGTTCCACT-3′) (Ruzek et al., 2007). The same PCR protocol as for amplification of spirochete DNA was used. Annealing temperature of the primers was set to 50 °C. Positive and negative controls were included for reverse transcription and PCR. The PCR products were analyzed by agarose gel electrophoresis.

From the *Borrelia* positive samples, 267 were semi-randomly selected (with respect to representation of different genospecies) for quantification of copies of borrelial genome by real-time PCR (targeting a portion of the *flagellin* gene of *B. burgdorferi*). The samples were analyzed in triplicates in 25 μl reactions consisting of 12.5 μl Probe qPCR Master Mix ($(NH_4)_2SO_4$, MgCl2, KCl, dNTP mix, Hot Start Taq DNA polymerase) (Fermentas), primer FlaF1 (5′-AGCAAATTTAGGTGCTTTCCAA-3′) (300 nM final concentration), primer FlaR1: (5′-GCAATCATTGCGATTGCAGA-3′) (900 nM final concentration), TaqMan probe FlaProbe1