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Short communication

Spatial distribution of *Anaplasma phagocytophilum* and *Hepatozoon canis* in red foxes (*Vulpes vulpes*) in Hungary

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ABSTRACT

In recent years, *Ehrlichia canis* and *Hepatozoon canis* transmitted by *Rhipicephalus sanguineus* were reported from Hungary. The aim of the present study was to reveal the spatial distribution pattern of pathogens transmitted by *R. sanguineus* in a sentinel species, red fox (*Vulpes vulpes*) in Hungary and to analyse the relationship of these patterns with landscape and climate by geographical information systems. Fox carcasses, representing 0.5% of the total fox population were randomly selected out of all the foxes of Hungary. The spleen samples of the animals were tested by real-time PCR for *Anaplasma platys*, *Babesia vogeli*, *E. canis* and *H. canis* infection. Positive results were confirmed by conventional PCR followed by sequencing. The prevalence of *H. canis* infection was 22.2% (95% CI = 18.4–26.4%), and this parasite was detected in all areas including the mountain regions of Hungary. These findings indicate that other tick species or other transmission routes (oral and transplacental) might be in the background of the countrywide distribution of *H. canis*. *Anaplasma platys* was not found; nevertheless, the prevalence of *Anaplasma phagocytophilum* infection transmitted by *Ixodes ricinus* was 12.5% (95% CI = 9.7–16.1%) in foxes. *B. vogeli* and *E. canis* infection was not detected. There was no correlation between environmental parameter values in the home range of foxes and *A. phagocytophilum* or *H. canis* infection, which is in line with that observed in the case of tick species infesting foxes in Hungary. The results of this study indicate that *R. sanguineus*, if present, might be rare in Hungary. Our baseline study can be used for future evaluation of the effect of climate change on the spreading and emergence of *R. sanguineus* transmitted pathogens in Hungary.

Introduction

Anaplasma platys, *Babesia vogeli*, *Ehrlichia canis* and *Hepatozoon canis* are the causative agents of infectious cyclic thrombocytopenia, babesiosis, monocytic ehrlichiosis and hepatozoonosis in wild canids and dogs. These pathogens have been reported worldwide and are transmitted by the brown dog tick (*Rhipicephalus sanguineus* sensu lato) (Dantas-Torres and Otranto, 2015). *R. sanguineus* is known to occur in the Mediterranean Basin of Europe (Estrada-Peña et al., 2013). In recent years, two dogs were serologically positive to *E. canis* in the southern part of Hungary and *E. canis* was detected in a small number of *Dermacentor marginatus* nymphs collected from dogs (Hornok et al., 2013a; Farkas et al., 2014a). Moreover, *H. canis* was also reported from dogs, red foxes (*Vulpes vulpes*) and golden jackals (*Canis aureus*) in the southern part of Hungary (Hornok et al., 2013b; Farkas et al., 2014b). These findings may indicate that climate change resulted in the spreading and emergence of *R. sanguineus* in a country with typical continental climate (Farkas et al., 2014b). The aim of the present study was to reveal the spatial distribution of pathogens transmitted by *R. sanguineus* in a sentinel species, red fox in Hungary and to analyse the relationship of the distribution patterns of these infections with landscape and climate by geographical information systems.

Materials and methods

Sample collection

Carcasses of red foxes sent to the National Food Chain Safety Office, Budapest, from November 2013 to June 2014, in connection with the rabies, *Trichinella* and *Echinococcus multilocularis* monitoring programmes, were included in this study. Carcasses were

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forwarded at +4 °C in individual plastic bags labelled by the hunters with an identification number reporting the information on the nearest place to killing on the topographic map and the date of hunting. If the nearest place to hunting was a human settlement, the fox position within a municipality was randomly chosen as described by Conraths et al. (2003). The fox sample size used in this study was previously calculated on the basis of the fox population size of each county described in the National Game Management Database (http://www.vvt.gau.hu/vadgazdalkodasi_statisztikak.htm). Fox carcasses (n = 415), representing 0.5% of the total fox population of each county, were randomly selected out of all the foxes from 19 counties and from the Budapest municipality (covering 100% of the Hungarian territory, 93,029 km^2). At necropsy, spleen was removed, a spleen fragment was collected using a sterile forceps and scissors, and then the fragment was stored in an Eppendorf tube at −20 °C until DNA isolation.

DNA isolation, PCR and sequencing

Frozen spleen fragments from foxes were thawed, and DNA was extracted from a 20 mg section using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) in accordance with manufacturer's protocol. The quality and concentration of each DNA sample was determined using a digital spectrophotometer (NanoDrop® ND-1000, Thermo Scientific, Wilmington, USA). The DNA samples were then adjusted to concentration of 150 ng/μl, aliquoted and stored at −20 °C until real-time PCR assays were performed. Real-time PCR assays for the detection of *A. platys*, *B. vogeli*, *E. canis* and *H. canis* were performed as described by other authors (Baneth et al., 2009; Costa-Júnior et al., 2012; Cabello et al., 2013; Ramos et al., 2014). The assays targeted 16S rDNA of A. *platys* and *E. canis*, and 18S rDNA of *B. vogeli* and *H. canis*. The protocols of the assays were not modified. Positive results were also confirmed by conventional PCR followed by sequencing of a 382 bp long fragment of 16S rDNA of *Anaplasma* and a 666 bp long fragment of 18S rDNA of *Hepatozoon* spp. (Santos et al., 2009; Otranto et al., 2011). Sequencing of *Anaplasma* products revealed the lack of species specificity of the real-time PCR used in the present study. Amplifications were done using CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, USA) with SsoFast Probes Supermix (Bio-Rad), SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire). Sequences were identified by comparison with GenBank entries using the BLAST program.

Geographic information system database, spatial and statistical analysis

The locality of origin of foxes and the infection status were marked on a point layer by the Quantum GIS 2.2 software (QGIS Team, 2012). The vector layers of altitude, mean annual temperature, annual precipitation, land cover, permanent water bodies were obtained from VÁTI Hungarian Nonprofit Ltd. (Budapest, Hungary) or created by Quantum GIS 2.2 on the basis of the georeferenced digital maps of the Hungarian Meteorological Service. The resolution of vector layers was 10–100 m. Along permanent water bodies (drinking and resting places of animals), a 100 m broad buffer zone was created, where the probability of the presence of host of ticks is high. The radius around the topographic position of foxes was restricted to 2.5 km, which was assumed to represent the average home range of foxes (Staubach et al., 2001). The digitized home ranges and the vector data were used to calculate the altitude, mean annual temperature, annual precipitation, areas of land cover types and the presence and buffer zone of permanent water bodies within the fox territories by Quantum GIS 2.2. Logistic regression analysis was performed with environmental parameter values and *Anaplasma phagocytophilum* and *H. canis* infection to identify the environmental conditions which affected the prevalence of these pathogens in Hungary. Comparison of the prevalence observed in this and other studies were compared by Fisher's exact test. Statistical analyses were carried out with MedCalc 12.7 (MedCalc Software, Ostend, Belgium).

Table 1
Prevalence of *Anaplasma phagocytophilum* and *Hepatozoon canis* in the spleen samples of 415 red foxes (*Vulpes vulpes*).

Counties	Prevalence (95% confidence interval)	
	A. phagocytophilum	*H. canis*
Bács-Kiskun (n = 25)	4.0 (1.0–19.5)	8.0 (2.0–25.0)
Baranya (n = 23)	8.7 (2.4–26.8)	21.7 (9.7–41.9)
Békés (n = 19)	5.3 (0.1–26.0)	15.8 (5.5–37.6)
Borsod-Abaúj-Zemlén (n = 24)	12.5 (4.3–31.0)	20.8 (9.2–40.5)
Csongrád (n = 19)	5.3 (0.1–26.0)	15.8 (5.5–37.6)
Fejér (n = 22)	18.2 (7.3–38.5)	18.2 (7.3–38.5)
Győr-Sopron (n = 18)	27.8 (12.5–50.9)	27.8 (12.5–50.9)
Hajdú-Bihar (n = 25)	4.0 (1.0–19.5)	8.0 (2.0–25.0)
Heves (n = 18)	11.1 (3.1–32.8)	16.7 (5.8–39.2)
Jász-Nagykun-Szolnok (n = 20)	10.0 (2.8–30.1)	15.0 (5.2–36.0)
Komárom-Esztergom (n = 10)	20.0 (5.7–51.0)	50.0 (23.7–76.3)
Nógrád (n = 13)	23.1 (5.0–53.8)	53.8 (29.1–76.8)
Pest including Budapest (n = 31)	16.1 (7.1–32.6)	12.9 (5.1–28.9)
Somogy (n = 37)	10.8 (4.3–24.7)	35.1 (21.8–51.2)
Szabolcs-Szatmár-Bereg (n = 25)	4.0 (1.0–19.5)	28.0 (14.3–47.6)
Tolna (n = 19)	15.8 (5.5–37.6)	42.1 (23.1–63.7)
Vas (n = 15)	13.3 (3.7–33.9)	20.0 (7.0–45.2)
Veszprém (n = 26)	19.2 (8.5–37.9)	19.2 (8.5–37.9)
Zala (n = 26)	19.2 (8.5–37.9)	19.2 (8.5–37.9)
Average (n = 415)	12.5 (9.7–16.1)	22.2 (18.4–26.4)

Results and discussion

The prevalence of *H. canis* infection was 22.2% in foxes (Table 1). The sequences obtained from Hungarian foxes were 99–100% identical to *H. canis* sequences obtained from Central European dogs and foxes and deposited in the GenBank (accession nos. FJ497012, FJ497019, GU371448, HM212625, KC584773, KJ572978 and KM096413). The parasite was widely distributed in all areas including the mountain ranges of Hungary (Fig. 1 and Table 1); nevertheless, the prevalence was considerably lower in the lowland regions of the country (Fig. 1). The parasite was detected earlier only in the southern region of Hungary with significantly lower prevalence (7.8%, 95% CI = 5.4–11.2%) (Farkas et al., 2014b). The detection of autochthonous hepatozoonosis in Hungary might imply that the range of *R. sanguineus* has reached the country (Farkas et al., 2014b). Nevertheless, *R. sanguineus* has never been found on wild canids and dogs or vegetation in Hungary (Földvári and Farkas, 2005; Sréter et al., 2005; Széll et al., 2006; Hornok and Farkas, 2009; Hornok et al., 2014). Our data indicate that *H. canis* is more widely distributed in Hungary than previously thought (Farkas et al., 2014b), and the prevalence is relatively high in the mountain regions of the country with cool climate (Fig. 1). The spatial distribution of *H. canis* indicates that other tick vectors may be in the background of the countrywide distribution of *H. canis* (Giannelli et al., 2013; Najm et al., 2014). Moreover, as oral and transplacental transmission of the parasite were also demonstrated (Baneth et al., 2001), *H. canis* might spread within the fox population without the involvement of tick hosts. It may also explain the presence of *H. canis* in Austria and Slovakia bordering Hungary on the north and west (Majláthová et al., 2007; Duscher et al., 2014). The infection rate of foxes was significantly lower than that seen in Austria (58.3%, 95% CI = 42.2–72.9%; n = 36) and Germany (45.2%, 95% CI = 39.3–51.3%; n = 261), similar to that observed in Croatia (23.0% 95% CI = 17.6–29.5%; n = 191) and significantly higher than that detected in Poland (11.6%, 95% = 7.0–19.0%; n = 111) (Dezdek