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A molecular survey of *Babesia* spp. and *Theileria* spp. in red foxes (*Vulpes vulpes*) and their ticks from Thuringia, Germany

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ABSTRACT

Wild canines which are closely related to dogs constitute a potential reservoir for haemoparasites by both hosting tick species that infest dogs and harbouring tick-transmitted canine haemoparasites. In this study, the prevalence of *Babesia* spp. and *Theileria* spp. was investigated in German red foxes (*Vulpes vulpes*) and their ticks.

DNA extracts of 261 spleen samples and 1953 ticks included 4 tick species: *Ixodes ricinus* ($n = 870$), *I. canisuga* ($n = 585$), *I. hexagonus* ($n = 485$), and *Dermacentor reticulatus* ($n = 13$) were examined for the presence of *Babesia*/*Theileria* spp. by a conventional PCR targeting the *18S rRNA* gene.

One hundred twenty-one out of 261 foxes (46.4%) were PCR-positive. Out of them, 44 samples were sequenced, and all sequences had 100% similarity to *Theileria annae*. Similarly, sequencing was carried out for 65 out of 118 PCR-positive ticks. *Theileria annae* DNA was detected in 61.5% of the sequenced samples, *Babesia microti* DNA was found in 9.2%, and *Babesia venatorum* in 7.6% of the sequenced samples. The foxes were most positive in June and October, whereas the peak of tick positivity was in October. Furthermore, the positivity of the ticks was higher for *I. canisuga* in comparison to the other tick species and for nymphs in comparison to adults.

The high prevalence of *T. annae* DNA in red foxes in this study suggests a reservoir function of those animals for *T. annae*. To our knowledge, this is the first report of *T. annae* in foxes from Germany as well as the first detection of *T. annae* and *B. microti* in the fox tick *I. canisuga*.

Detection of DNA of *T. annae* and *B. microti* in three tick species collected from foxes adds new potential vectors for these two pathogens and suggests a potential role of the red fox in their natural endemic cycles.

Introduction

Babesiosis, caused by haematozoan organisms infecting erythrocytes and belonging to the genus *Babesia*, is a tick-borne disease affecting a wide range of domestic and wild animals and occasionally man (Vannier and Krause, 2009). Cases of human babesiosis in Europe are rare and mainly assigned to *B. divergens*, but cases caused by *B. venatorum* (previously *Babesia* sp. EU1) or by *B. microti* have also been described (Herwaldt et al., 2003; Hildebrandt et al., 2007). *Babesia microti* and *Babesia duncani* are mainly responsible for human babesiosis in North America (Vannier and Krause, 2009).

Babesia venatorum was detected in humans for the first time in Italy and Austria (Herwaldt et al., 2003). It has also been identified in roe deer, the proposed reservoir host of this parasite (Bonnet et al., 2007, 2009; Tampieri et al., 2008), and in reindeer (Kik et al., 2011). *Babesia venatorum* DNA has also been detected in *Ixodes ricinus* ticks collected from animals (Lempereur et al., 2011, 2012; Burri et al., 2011) as well as in questing *I. ricinus* ticks (Schorn et al., 2011; Gigandet et al., 2011; Reis et al., 2011) and in engorged *Haemaphysalis punctata* (Hilpertshauser et al., 2006).

Babesia microti is a rodent-associated blood protozoan parasite that was first reported as a cause of human disease in 1969 in the north-eastern United States (Western et al., 1970), where *Ixodes*

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scapularis is supposed to serve as a vector (Spielman, 1976). In Europe, the mouse tick *Ixodes trianguliceps* seems to be the main vector for *B. microti*. However, the European tick *I. ricinus* was experimentally proved to be an efficient vector of *B. microti*, too (Gray et al., 2002). *Babesia microti* DNA has been detected in *I. ricinus* ticks from Thuringia, Germany (Hildebrandt et al., 2010), as well as from other countries throughout Europe (Rudolf et al., 2005; Burri et al., 2011; Sytykiewicz et al., 2012) and more recently in questing adult *Dermacentor reticulatus* ticks for the first time in Europe (Wojcik-Fatla et al., 2012).

In the past, it was assumed that the only *Babesia* species causing disease in dogs were *B. canis* and *B. gibsoni*. However, Zahler et al. (2000) identified a new organism by using molecular biological methods, which appears to be closely related to *B. microti* and which has been denominated as *Theileria annae*. Subsequently, *T. annae* (also denominated as *Babesia microti*-like) was detected in north-western Spain in dogs manifesting intense anaemia, thrombocytopenia, and renal failure as common findings. It was suggested to be endemic in that area among the canine population (Camacho et al., 2001). In that area, the European hedgehog tick *I. hexagonus* was assumed to be the main vector of *T. annae* based mainly on detecting only this tick species on all *Babesia*-positive dogs (Camacho et al., 2003). In addition to *I. hexagonus*, *T. annae* has been detected in *I. ricinus* and *Rhipicephalus sanguineus* ticks (Lledo et al., 2010; Iori et al., 2010).

In addition to dogs, molecular studies have identified *T. annae* DNA in cats (10%), in a roe deer, a donkey, in horses (1.2%), as well as in red foxes from southern Europe (5–69.2%) (Criado-Fornelio et al., 2003, 2007; Tampieri et al., 2008; Gimenez et al., 2009; Moretti et al., 2010; Dezdek et al., 2010; Cardoso et al., 2013).

Red foxes live in proximity to humans and domestic dogs, thus they constitute a possible source of veterinary and zoonotic diseases (Fishman et al., 2004).

To our knowledge there is no information available about babesial/theilerial infections in red foxes and their ticks in Germany, thus the aim of this study was to investigate the prevalence of such infections in red foxes from Thuringia, Germany, and their ticks by means of molecular techniques.

Materials and methods

Spleen and tick samples

All samples included in the present study were collected during a previous study, in which 1286 carcasses of red foxes from Thuringia, Germany, had been examined between January 1st and December 31st, 2009, for their infestations with ticks. Identification of collected ticks resulted in 4 species (*I. ricinus*, *I. canisuga*, *I. hexagonus*, and *D. reticulatus*). Spleen samples were taken from most of the examined foxes (Meyer-Kayser et al., 2012). Of the previous study, a total of 267 foxes included all foxes which were infested with 2 or 3 tick species ($n = 233$), as well as 34 foxes infested with only one tick species and distributed almost equally over the year were chosen for this study. Out of 3340 ticks, which had been collected from these foxes, 1953 ticks (specified as engorged ticks) have been included in this study. Species and the level of engorgement of the ticks had previously been determined (Meyer-Kayser et al., 2012). A maximum of 5 ticks per species or stage were chosen from each individual fox. From 6 of the chosen foxes, no spleen sample was available. Tick abundance was as follows: 870 *I. ricinus* (560 females, 235 males, and 75 nymphs), 585 *I. canisuga* (155 females and 430 nymphs), 485 *I. hexagonus* (198 females, 3 males, and 284 nymphs), and 13 *D. reticulatus* (7 females and 6 males).

DNA extraction

DNA was extracted from the spleen tissues and the ticks using the Maxwell® 16 LEV blood DNA kit (Promega, Madison, USA). Up to 50 mg of spleen tissue were incubated with 30 μl proteinase K and 300 μl lysis buffer at 56 °C overnight, whereas the whole tick, after disrupting it in a bead-beating tissue lyser (Qiagen, Germany) for 5 min with 300 μl phosphate buffered saline (PBS), was incubated under the same conditions for 2 h. The lysate was then processed in a Maxwell® 16 automated DNA extraction instrument (Promega). Elution was done with 50 μl elution buffer. Amount and quality of extracted DNA were measured with NanoDrop® 1000 (PeqLab, Erlangen, Germany), and DNA samples were stored at −20 °C until use.

Polymerase chain reaction (PCR) screening

PCR screening of spleens and tick samples for *Babesia/Theileria* DNA was performed using the following primers: BJ1 (5′-GTC TTG TAA TTG GAA TGA TGG-3′) and BN2 (5′-TAG TTT ATG GTT AGG ACT ACG-3′), which were designed on the basis of the common sequence of the *18S rRNA* gene of several *Babesia* species (Casati et al., 2006). According to the sequence of the *18S rRNA* gene of *B. divergens* (AJ439713), the position of the amplified fragment is 488–912 (Casati et al., 2006). Amplifications were performed in 50-μl reactions containing 5 μl of buffer (10× concentrated contains Tris–Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7) with 0.2 mM of each deoxynucleoside triphosphate, 1.25 U of Taq polymerase, 1 μM of each primer, 5 μl of DNA sample, and made up to 50 μl with deionized PCR clean water. The mixtures were subjected to an initial denaturation step of 95 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 40 s. Amplification was completed by a further step of 5 min at 72 °C. PCR products were separated by electrophoresis in 2% agarose gels stained with GelRed™ (Biotium, Hayward, USA). Amplified samples were purified using the QIAquick purification kit (Qiagen, Hilden, Germany) and sequenced by a commercial company (Eurofins MWG Operon, Ebersberg, Germany). The sequences were evaluated using Chromas Lite (www.technelysium.com.au/chromas_lite.html) and compared to sequences deposited in GenBank with BLASTn.

Statistical analysis

A semiparametric logistic regression model (Wood, 2006) was used to test the effect of different tick parameters (total number of ticks and number of tick species present on each individual fox) on the probability of babesial/theilerial infections of fox. In each model, sex (male, female), age (<1 yr, 1–2 yrs, 2–3 yrs, >3 yrs), and feeding status (thin, normal, very good) of foxes was tested, and a cyclic cubic regression spline was included in all models for month. It was additionally investigated whether (i) the total number of positive ticks, (ii) the number of every developmental stage (adults/nymphs) of positive ticks, (iii) the number of ticks of a particular species or of a combination of species, has an effect on the positivity of each individual fox for *Babesia/Theileria* spp. In order to estimate the effect of species, development stages (adult/nymph), fox positivity, and time of hunting of the fox (month) on the positivity of ticks, a semiparametric logistic regression model was also conducted. The *D. reticulatus* ticks were omitted in the species-specific analysis due to the low number of this tick species. $p < 0.05$ was regarded as significant. Statistical analysis was performed with R version 2.15.1 (R Development Core Team, 2012).

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