Fox on the run – molecular surveillance of fox blood and tissue for the occurrence of tick-borne pathogens in Austria

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Abstract

Background: The red fox (Vulpes vulpes) is a widespread species, harbouring many pathogens relevant for humans and pets. Indeed, Anaplasma spp., Ehrlichia canis and Rickettsia spp. among the bacteria and Hepatozoon canis as well as Babesia sp. among the parasites have been the focus of several studies.

Findings: In a cohort of 36 foxes shot on one day in the north-eastern part of Austria, Babesia microti-like pathogens were found in 50%, while H. canis was detected in 58.3% of the samples. The spleen was more useful for detection of H. canis, whereas B. microti-like parasites were more frequently found in the blood. Bacteria could not be confirmed in any of the cases to demonstrate the occurrence of such tick-borne pathogens using PCR and sequencing on blood and spleen samples.

Conclusions: The occurrence of B. microti-like and H. canis parasites raised many questions, because these infections have never been found autochthonously in dogs. Furthermore in the case of H. canis the main vector tick, Rhipicephalus sanguineus, is absent in the sampling area, leaving space for further hypotheses for transmission such as vertical transmission, transmission via ingestion of prey animals or other vector ticks. Further studies are needed to evaluate the risks for pets in this area. PCRs delivered differing results with the different tissues, suggesting the use of both spleen and blood to obtain an integral result.

Keywords: Hepatozoon canis, Anaplasma phagocytophilum, Babesia microti-like

Findings

Background

Red foxes (Vulpes vulpes) are among the most widely distributed mammals in the world and are invading many urban areas due to a good adaptation to human environments, and to rabies vaccination [1]. As a result foxes might play a big role in spreading pet-relevant pathogens and parasites such as mites and ticks [2]. Recently they have been discussed as a potential reservoir for blood parasites like Anaplasma phagocytophilum [3], Hepatozoon canis [4], Babesia sp. [5], Ehrlichia canis [6] and Rickettsia spp. [2]. Due to their close vicinity to domestic habitats they may act as a transmission interface for some of these pathogens to pets and humans [5].

Babesia microti-like parasites – also known as Babesia sp., Babesia annae or Theileria annae – are frequently found in foxes in countries such as Croatia [7], Portugal [5] and Spain [8]. The common assumption is that Ixodes hexagonus is involved in the transmission cycle [9], and a recent study identified I. ricinus and I. canisuga as carriers and therefore as potential vectors [10]. These ticks could also serve as a transmission interface to dogs, where Babesia may cause azotaemia, haemolytic anaemia, renal failure and mortality [11].

Hepatozoon canis affects canids and its occurrence is mostly linked to the distribution of the main vector tick Rhipicephalus sanguineus [12], already displaying exceptions in countries such as Austria, Germany or Hungary [12-14].

The aim of this study is to evaluate the role of foxes in terms of their blood pathogens and to discover potential reservoirs for tick-borne diseases in northern latitudes.
Method
Foxes shot on 18 January 2014 in the district of Gänserndorf (in the northeast of Lower Austria) were further processed on the same day. From the 36 foxes, 35 spleen samples and 17 blood samples were obtained. Extraction of DNA from blood and tissue was performed as previously described [14]. Primers detecting Anaplasma sp., Babesia sp. (piroplasms), Ehrlichia canis, Hepatozoon canis and Rickettsia sp. were used (Table 1). The PCRs were conducted on the Eppendorf Mastercycler pro S (Eppendorf AG, Hamburg, Germany) using protocols published elsewhere [14]. To confirm the sequence, positive samples were randomly chosen and the amplifications were purified by Fast-kit (Bio-Rad Laboratories, Vienna, Austria) according to the manufacturer's recommendations and sent for sequencing (Microsynth AG, Balgach, Switzerland; LGC, Teddington, UK). Sequences obtained were further processed by GeneDoc (http://gene.doc.software.informer.com/2.7/) and blasted on GenBank® (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Ethical statement
Fox were shot during routine hunting events under the restrictions of the game laws of the province of Lower Austria.

Results
The investigation of the blood and spleen samples identified 18 B. microti-like pathogen-positive foxes, 21 foxes harbouring H. canis and four foxes with double infections (Table 2), leading to prevalences of 50%, 58.3% and 11.1%, respectively. PCRs for detecting piroplasms (Babesia sp. nested) in blood and spleen detected 13 (76.5% of the blood samples) and 11 (31.4% of the spleens) B. microti-like pathogens, respectively. Sequences of these pathogens showed 98–100% similarity to B. sp. “Spanish dog” (e.g. GenBank® accession no. AF188001.1 or EU58387.1). Using the Hepatozoon-specific primers, 21 foxes tested positive for H. canis. The investigation of the spleen samples identified 18 positive results (51.4%), whereas in the blood samples only six positive results (35.3%) were found. Seven more PCR products, positive on the gel, provided no conclusive sequence data, and therefore were noted as false positives. All conclusive sequences delivered 99–100% similarity to H. canis found in GenBank® (e.g. accession no. AY150067.2, DQ111754.1, JN584477.1 or KC509526.1).

In none of the blood or spleen samples could Anaplasma sp., E. canis or Rickettsia spp. be detected.

Discussion
Foxes are known to be major reservoirs for Babesia microti-like parasites [5]. The high prevalence of 50% found in this study and in this population is therefore not surprising and reflects a similar situation in Germany with 46.4% [10], Portugal with 69.2% [5] and Spain with 14% to 50% [8].

The 58.3% positive H. canis foxes in Austria are in concordance with four positive foxes out of nine found in Slovakia [19], 45.2% in Germany [20], 16 out of 111 investigated foxes (11.6%) in Poland [21] or 8% in Hungary [22]. To date H. canis is not found endemically in dogs in these areas, nor is R. sanguineus known to occur autochthonously [12,19,21,23], although H. canis has already been found in dogs in areas lacking the main vector tick in Germany [13,20] and Hungary [12,22].

Table 1 PCR parameters for amplification of DNA of target organisms

| Target organism | Forward primer (5'-3') | Reverse primer (5'-3') | No. of cycles | Annealing temperature (°C) | Primer concentration (pmol) | Product size (bp) | Reference |
|-----------------|------------------------|------------------------|--------------|--------------------------|---------------------------|-----------------|----------|
| Anaplasma sp.   | Ehr.u.for: GTT TGA TCC TGG CTC AGG AYD AAC | Ehr2rev: CTC TTT CGA CCT CTA GTC TAG C | 30           | 66.8                     | 12.5                      | 619             | [15]     |
| Piromas (nested) | BTH-1 F: CCT GAG AAA CGG CTA CCA CAT CT | BTH-1R: TTG CGA CCA TAC TCC CCC CA | 40           | 68                       | 25                        | 561             | [16]     |
|                 | 2nd                    | GR2: CCA AAG ACT TTG ATT TCT CTC | 40           | 60                       | 5                          | 561             | [16]     |
| Ehrlichia canis | Ehr.u.for: GTT TGA TCC TGG CTC AGG AYD AAC | Ehr.CCE.rev: CTC TTT CGA CCT CTA GTC TAG C | 30           | 65.0                     | 20                        | 619             | [15]     |
| Hepatozoon canis | HEPF: ATA CAT GAG CAA AAT CTC AAC | HEPR: CTT ATT ATT CCA TGC TGC AG | 35           | 57.0                     | 10                        | 660             | [17]     |
| Rickettsia sp.  | ITS-F: GAT AGG TCG GGT GTG GAA G | ITS-R: TCG GGA TGG VAT CGT GTG | 35           | 52                       | 1                         | 342 – 533       | [18]     |
Conclusion

Foxes represent a good reservoir for several zoonotic and pet-relevant diseases. In terms of blood parasites this seems more the rule than the exception. Human- and pet-relevant agents such as *Babesia microti*-like pathogens and *H. canis* could be found in a relatively small set of fox samples originating from north-eastern Austria. Especially, the occurrence of *H. canis* in considerable numbers in this population so far north raises many questions such as the potential impact on domestic animals, reservoirs

| Fox | PCR | Piroplasms nested | H. canis | Pathogens detected | GenBank® accession no |
|-----|-----|------------------|---------|-------------------|-----------------------|
| 1   |     | *B. microti*-like | pos. f  | *B. microti*-like | KM115968              |
| 2   |     | *H. canis*       | *H. canis* | *H. canis* | KM115969              |
| 3   |     | *H. canis*       | pos.     | *H. canis* | KM115970              |
| 4   |     | *H. canis*       | *H. canis* | *H. canis* | KM115971              |
| 5   |     | *B. microti*-like | neg.     | *B. microti*-like | KM115972              |
| 6   |     | *B. microti*-like | pos. f  | *B. microti*-like | KM115973              |
| 7   |     | *H. canis*       | *H. canis* | *H. canis* | KM115974              |
| 8   |     | *B. microti*-like | neg.     | *B. microti*-like | KM115975              |
| 9   |     | *B. microti*-like | neg.     | *B. microti*-like | KM115976              |
| 10  |     | pos              | pos. f  | unclear           |                       |
| 11  |     | *B. microti*-like | pos. f  | *B. microti*-like | KM115977              |
| 12  |     | *B. microti*-like | neg.     | *B. microti*-like | KM115978              |
| 13  |     | *H. canis*       | *H. canis* | *H. canis* | KM115979              |
| 14  |     | *B. microti*-like | *H. canis* | *H. canis* | KM115980/KM115981     |
| 15  |     | *B. microti*-like | pos.     | *B. microti*-like | KM115982/KM115983     |
| 16  |     | *H. canis*       | *H. canis* | *H. canis* | KM115984              |
| 17  |     | *B. microti*-like | pos. f  | *B. microti*-like | KM115985              |
| 18  |     | *H. canis*       | *H. canis* | *H. canis* | KM115986              |
| 19  |     | *H. canis*       | *H. canis* | *H. canis* | KM115987              |
| 20  |     | *B. microti*-like | *H. canis* | *H. canis* | KM115988/KM115989     |
| 21  |     | *B. microti*-like | neg.     | *B. microti*-like | KM115990              |
| 22  |     | *H. canis*       | *H. canis* | *H. canis* | KM115991              |
| 23  |     | *B. microti*-like | neg.     | *B. microti*-like | KM115992              |
| 24  |     | *H. canis*       | *H. canis* | *H. canis* | KM115993              |
| 25  |     | *B. microti*-like | neg.     | *B. microti*-like | KM115994              |
| 26  |     | *H. canis*       | *H. canis* | *H. canis* | KM115995              |
| 27  |     | *H. canis*       | *H. canis* | *H. canis* | KM115996              |
| 28  |     | *B. microti*-like | pos. f  | *B. microti*-like | KM115997              |
| 29  |     | *H. canis*       | *H. canis* | *H. canis* | KM115998              |
| 30  |     | *B. microti*-like | *H. canis* | *H. canis* | KM115999/KM116000     |
| 31  |     | *H. canis*       | *H. canis* | *H. canis* | KM116001              |
| 32  |     | pos.             | *H. canis* | *H. canis* | KM116002              |
| 33  |     | *H. canis*       | *H. canis* | *H. canis* | KM116003              |
| 34  |     | *B. microti*-like | neg.     | *B. microti*-like | KM116004              |
| 35  |     | *H. canis*       | *H. canis* | *H. canis* | KM116005              |
| 36  |     | *B. microti*-like | pos. f  | *B. microti*-like | KM116006              |

Table 2 PCR results of spleen and blood compared to sequencing results of the investigated foxes (pos = representing a positive PCR product on the gel, neg = delivering no band on the gel, *H. canis* or *B. microti*-like = confirmed sequence of this pathogen in the substrate, “f” indicates false positive samples showing a gel band, but not confirmed during sequencing)
and infection pathways. Moreover, the main vector tick, *Rhipicephalus sanguineus*, is absent in the sampling area. Therefore other transmission pathways such as vertical transmission, transmission via ingestion of preyed animals or other vector ticks need to be evaluated.

Thus foxes have to be considered during treatment strategies and *B. microti*-like as well as *H. canis* pathogens have to be recognized as an unnoticed threat in northern areas. The use of piroplasm PCRs could help to identify both *B. microti*-like and *H. canis* pathogens prior to screening, followed by PCRs with species-specific primers.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

GGD organized PCR on the samples and wrote the manuscript, HPF performed sequence analysis and AKH took the samples and organized the study. All authors read and approved the manuscript.

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