Veterinary Parasitology 224 (2016) 44–51

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Vector-borne pathogens in dogs and red foxes from the federal state of Brandenburg, Germany

Jana M. Liesner[a], Jürgen Krücken[a], Roland Schaper[b], Stefan Pachnicke[c], Barbara Kohn[d], Elisabeth Müller[e], Christoph Schulze[f], Georg von Samson-Himmelstjerna[a,*]

[a] Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Robert-von-Ostertag Str. 7-13, 14163 Berlin, Germany
[b] Bayer Animal Health GmbH, Alfred-Nobel-Str. 50, Monheim, 40789, Germany
[c] Bayer Vital GmbH, Kaiser-Wilhelm-Allee 70, 51373 Leverkusen, Germany
[d] Small Animal Clinic, Freie Universität Berlin, Oertzenweg 19, 14163 Berlin, Germany
[e] LABOKLIN GmbH & Co. KG, Steubenstr. 4, Bad Kissingen, 97688, Germany
[f] Landeslabor Berlin-Brandenburg, Gerhard-Neumann-Str. 2, Frankfurt (Oder), 15236, Germany

ARTICLE INFO

Article history:
Received 28 February 2016
Received in revised form 8 May 2016
Accepted 10 May 2016

Keywords:
Canine vector-borne diseases
Zoonosis
Dirofilariosis
Babesiosis
Anaplasmosis
Neoehrlichiosis
Rickettsiosis

ABSTRACT

Dirofilaria repens is endemic in eastern and southern European regions but was recently found in Germany in dogs, mosquitoes and one human patient. Since some of the positive dog and mosquito samples were collected in Brandenburg, it was aimed to systematically assess the prevalence of *D. repens* and other canine vector-borne pathogens in Brandenburg. Dog owners also received a questionnaire and were asked to provide more information about the dogs including travel history. In total, 1023 dog blood samples as well as 195 fox spleen and 179 fox blood samples were collected. DNA was analysed by PCR for the presence of filariae, piroplasms, anaplasmataceae and *Rickettsia* spp. Filariae were detected in six dogs (0.6%), two were positive for DNA from *D. repens*, two from *Dirofilaria immitis* and two from *Acanthocheilonema reconditum*. One of the *D. repens* positive dogs originated from an animal shelter in Brandenburg, but the origin of the other one remained unknown. Interestingly, both *D. repens* ITS-1 sequences showed 100% identity to a *D. repens* sample obtained from a Japanese woman that travelled in Europe and were 97% identical to a newly proposed species *Dirofilaria* sp. 'hongkongensis' described from Hong Kong. However, identity to other *D. repens* sequences from Thailand was considerably lower (81%). Identity of 12S rRNA and cytochrome oxidase I to *D. repens* samples from southern Europe was 99%. Due to the low number of *Dirofilaria* spp. positive dogs and since the origin of these was unknown, endemic occurrence of *Dirofilaria* in Brandenburg could not be confirmed. *Anaplasma phagocytophilum* was found in 15 dogs (1.5%), *Candidatus* Neoehrlichia mikurensis in three dogs (0.3%) and *E. canis* in one dog (0.1%), which was co-infected with *D. repens*. *Rickettsia* spp. were detected in 8 dogs (0.8%), seven were *Rickettsia raoultii* and one was *Rickettsia felis*. To the author's knowledge, *R. raoultii* DNA was detected for the first time in dogs in Germany in this study and *Candidatus* N. mikurensis for the second time. In spleen samples of red foxes with 47.5% a high prevalence of piroplasms was found. Sequencing of 11 samples identified 10 as *Theileria annae*. Despite the high prevalence of this pathogen in its reservoir host, it was absent in dog samples. In one dog (0.1%), *Babesia canis* was detected but there was no further information about the dog's origin. Evaluation of the questionnaire identified a high proportion of dogs (74.2%, n = 233) which was not protected by ectoparasiticides. Moreover, 21.2% (n = 236) of the dogs originated from inland or abroad shelters, and therefore might potentially come from areas endemic for dirofilariosis or babesiosis.

1. Introduction

So far, *Dirofilaria repens* is considered to be endemic in the eastern and southern regions of Europe but not in Germany. In Germany, a few autochthonous cases have been described in the South West (upper Rhine valley) (Pantchev et al., 2009). In 2007 and 2012, however, a possibly autochthonous outbreak of *D. repens*

* Corresponding author.
E-mail addresses: gvsamson@fu-berlin.de, samson.georg@fu-berlin.de (G. von Samson-Himmelstjerna).

http://dx.doi.org/10.1016/j.vetpar.2016.05.012
0304-4017/

was reported repeatedly in a sledge dog kennel in the federal state of Brandenburg, Germany (Sassnau et al., 2009; Sassnau et al., 2013). Furthermore, this zoonotic parasite as well as the heartworm *Dirofilaria immitis* were found in local mosquito species from Brandenburg in the years 2011 and 2012 (Czajka et al., 2014) and was very recently detected in a human from Saxony-Anhalt (neighbouring state to Brandenburg) without travel history into endemic areas (Tappe et al., 2014). Due to these findings, it is currently considered to be an emerging infectious disease in Germany. In its definite host, *D. repens* causes subcutaneous skin nodules with itching, inflammation and redness or even no clinical signs at all. Regarding the therapy of dirofilariosis, efficacy of a combination of Imidacloprid and moxidectin against adult worms was recently proven for *D. repens* in experimentally infected dogs (Petry et al., 2015).

Other vector-borne pathogens such as Anaplasmataceae, *Babesia* spp., *Borrelia* spp. and *Rickettsia* spp. have recently been described with different frequency levels in ticks collected from dogs in Berlin and Brandenburg (Schreiber et al., 2014). For a recent review of these pathogens in dogs from Germany and Austria see Pantchev et al. (2015). Some members of the Anaplasmataceae are also known to occur in Germany (Kohn et al., 2011) or are frequently introduced by import of infected dogs (Menn et al., 2010; Rohrig et al., 2011). Seroprevalence of *Anaplasma phagocytophilum* is known to be very high in the region but frequency of dogs with ongoing infection as detected by PCR was very low (Kohn et al., 2011). *Candidatus* Neoehrlichia mikurensis is considered to be an emerging zoonotic pathogen transmitted by ticks of the genus *Ixodes* (Rizzoli et al., 2014). In the study area it has recently been described in rodents and *Ixodes ricinus* as well as *Ixodes hexagonus* ticks collected from dogs (Krücken et al., 2013; Schreiber et al., 2014). It is currently known to be among the most prevalent tick-borne pathogens in central Europe (Richter and Matuschka, 2012; Silaghi et al., 2015) and can rarely cause severe disease in dogs (Diniz et al., 2011) as well as humans (Fehr et al., 2010; Pekova et al., 2011; von Loewenich et al., 2010; Wenneras, 2015). In contrast to *A. phagocytophilum* and *Candidatus* N. mikurensis, *Ehrlichia canis* is not zoonotic, is transmitted by *Rhipicephalus sanguineus* and is widely distributed in the Mediterranean area (Trotz-Williams and Trees, 2003). However, it is frequently observed outside of its endemic region due to import of stray dogs into Central and Northern European countries including Germany (Menn et al., 2010).

Rickettsia spp. have been demonstrated to be the most frequently occurring pathogens in dog-associated ticks of the species *I. ricinus*, *I. hexagonus* and *Dermacentor reticulatus* in the Berlin/Brandenburg area (Schreiber et al., 2014). The pathogenicity of *Rickettsia conorii*, the causative agent of Mediterranean Spotted Fever, is well described (Colomba et al., 2006; Solano-Gallego et al., 2015). However, this pathogen is transmitted by *Rhipicephalus sanguineus*, a tick species which is found in Germany only occasionally in indoor habitats. The species frequently found in *Ixodes* spp. and *D. reticulatus*, the most abundant ticks in Berlin/Brandenburg are *Rickettsia helvetica* and *Rickettsia raoulti*, respectively. These *Rickettsia* have only rarely been described as causative agents of diseases but can cause perimyocarditis, meningitis (Fournier et al., 2000; Nilsson et al., 2010; Nilsson et al., 1999) and TIBOLA (tick-borne lymphadenopathy)/DEBONEL (*Dermacentor*-borne necrosis erythema lymphadenopathy) in humans (Oteo and Portillo, 2012; Parola et al., 2009).

To date, there are no data available of actual infection rates of dogs with vector-borne diseases from Brandenburg and the role of red foxes as potential reservoir hosts for these infections is also still unclear. Therefore, the aim of this study was to examine the epidemiological situation regarding the prevalence of *D. repens* and other filarioses, *A. phagocytophilum*, *Candidatus* N. mikurensis, *E. canis*, *Rickettsia* spp. and piroplasms in dogs and red foxes from Brandenburg. A questionnaire for owners of participating dogs aimed to provide information about origin, travel behaviour and parasite prevention and thus verify possible reasons for the spread of vector-borne diseases.

2. Materials and methods

2.1. Samples and study area

Canine blood samples (n = 1023) obtained from veterinary clinics or a commercial diagnostic laboratory originating from dogs from the federal state of Brandenburg, Germany, in the period between March 2013 and September 2014 were examined. All samples represented excess material from samples taken for other diagnostic purposes. Additionally, 179 blood and 195 spleen samples from red foxes from the federal state of Brandenburg were retrieved from February to September 2014 for further examination. These fox samples were obtained in the context of the German rabies surveillance program. The animals were hunted or killed in traffic accidents. No animal was killed with the aim of providing samples for this study and samples were not selected towards age, sex or other variables. However, it is unclear, if these samples are representative for the fox population in Brandenburg. DNA extraction followed by different polymerase chain reactions was performed for all the samples.

2.2. DNA extraction protocol

DNA extraction of the first 175 canine blood samples was performed using the Maxwell® 16 LEV Blood DNA Kit (Promega Corporation, USA) following the manufacturer's instructions. Presence of residues of paramagnetic cellulose particles led to the use of innuPREP Blood DNA mini Kit (Analytik Jena, Germany) for the rest of the samples. The manufacture's protocol was slightly modified in order to avoid filter plugging by diluting 100 μl blood with 100 μl 1 × PBS solution instead of the suggested use of 200 μl of blood. A different extraction kit, the innuSPEED Tissue DNA Kit (Analytik Jena, Germany) was used for spleen samples. Initial homogenization was required using the SpeedmillPLUS machine (Analytik Jena, Germany) followed by the DNA isolation with the innuSPEED Tissue DNA Kit according to the recommended protocol. DNA quality and quantity were finally measured using spectrophotometry in a Take3 plate and an Epoch® plate reader (BioTek Instruments, Inc., USA). DNA samples were stored at −20 °C for future use.

2.3. PCR protocols

The PCR protocol for the detection of *Dirofilaria* spp. targeting the internal transcribed spacer region 1 (ITS-1) was initially described by Nuchprayoon et al. (2005). Reactions contained 0.2 mM dNTPs (Thermo Fisher Scientific), 0.25 μM of each primer, 0.1 U/μl Phusion Hot Start II High Fidelity DNA Polymerase (Thermo Fisher Scientific) and 2 μl template DNA (20–100 ng/μl) in 20 μl 1 × Phusion HF buffer (Thermo Fisher Scientific). The detection limit of this PCR was 2 copies of plasmid DNA containing the target sequence. Cycler conditions included an initial denaturation step at 98 °C for 30 s, followed by 40 cycles at 98 °C for 10 s and 68 °C for 45 s, and a final elongation stage at 72 °C for 5 min. A plasmid (200 copies) containing an insert of an ITS-1 473 bp sequence from a non-described filarial species was used as a positive control in every PCR, whereas a reaction containing water instead of template DNA was employed as a negative control. According to GenBank entries, the expected amplicon sizes for *D. repens*, *D. immitis* and *Acanthocheilonema reconditium* were 595 bp, 599–602 bp and 446 bp, respectively. Positive samples were purified by Zymoclean™ DNA Recovery Kit (Zymo