ARTICLE IN PRESS

Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Original article

Detection of tick-borne pathogens in ticks from dogs and cats in different European countries

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ARTICLE INFO

Keywords:
Cat
Dog
Tick-Borne pathogens
Europe
Rhipicephalus sanguineus sp. II-temperate lineage
Haplotypes

ABSTRACT

Ticks are known to transmit pathogens which threaten the health and welfare of companion animals and man globally. In the present study, mainly adult ticks were collected from dogs and cats presented at their local veterinary practice in Hungary, France, Italy, Belgium (dogs only) and Germany (cats only), and identified based on tick morphology. If more than one tick was collected from a host animal, ticks were pooled by tick species for DNA extraction and subsequent PCR examination for the presence of tick-borne pathogens. Out of 448 tick samples, 247 (95 from dogs and 152 from cats) were *Ixodes ricinus*, 26 (12 from dogs and 14 from cats) were *I. hexagonus*, 59 (43 from dogs and 16 from cats) were *Dermacentor reticulatus* and 116 (74 from dogs and 42 from cats) were *Rhipicephalus sanguineus* sensu lato (s.l.). In 17% of the *I. ricinus* samples *Anaplasma phagocytophilum* was found. *Borrelia* spp. were mainly identified in *I. ricinus* collected from cats (18%) and to a lesser extent in dog-sourced ticks (1%), with *Borrelia afzelii* (n = 11), *B. garinii* (n = 7), *B. valaisiana* (n = 5), *B. lusitaniae* (n = 3) and *B. burgdorferi* sensu stricto (n = 3) being identified. One *I. hexagonus* sample collected from a cat in France tested positive for *B. afzelii*. *Babesia canis* was detected in 20% of the *D. reticulatus* samples, mainly from Hungary. *Rhipicephalus sanguineus* s.l. was found positive for *Hepatozoon canis* (3%), *A. platys* (5%) and three *Rickettsia* species (7%; *R. massiliae*; *R. raoultii* and *R. rhipicephali*). Furthermore, a total of 66 *R. sanguineus* s.l. ticks were subjected to molecular analysis and were identified as *R. sanguineus* sp. II-temperate lineage, with seven haplotypes recorded. Amongst them, the most prevalent sequence types were haplotype XIII (n = 24; 69%) and haplotype XIV (n = 16; 52%) in France and in Italy, respectively, found both in cats and dogs. Although differences related to both country and host, were observed, the results of this study indicate that cats and dogs are exposed to tick-borne pathogen infected ticks, which may represent a medical risk to these host animals.

1. Introduction

The most common ticks infesting dogs and cats in Europe are *Ixodes ricinus*, *I. hexagonus*, *Rhipicephalus sanguineus* sensu lato (s.l.) and *Dermacentor reticulatus*, with differences reported between countries regarding their occurrence. In addition to the potential direct clinical impact, ticks are also important vectors of different pathogens. For example, *Ixodes* ricinus is a known vector of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l. (respectively the pathogens causing granulocytic anaplasmosis and Lyme borreliosis). *Dermacentor reticulatus* is an important vector of *Babesia canis* which causes significant disease and mortality in dogs and *Rhipicephalus sanguineus* s.l. can transmit a range of pathogens including but not limited to *Anaplasma*, *Rickettsia*, *Babesia*, *Hepatozoon* and *Ehrlichia* spp. (Claerebout et al;, 2013; Dantas-Torres, 2008, 2012; Heyman et al., 2010; Parola et al., 2005; Solano-Gallego et al., 2016; Stich et al., 2008; Stuen et al., 2013).

The ability of ticks to transmit pathogens creates a persistent risk of vector-borne infections for human populations and domestic animals. Although tick infestations are commonly associated with rural areas, it has become increasingly clear that ticks are well adapted to urban and suburban environments (Rizzoli et al., 2014; Uspensky, 2014). In addition, some tick species expand their geographical distribution due to a number of abiotic and biotic factors, such as climate change, increased travel of dogs and cats with their owners, host expansion as well as

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https://doi.org/10.1016/j.ttbdis.2018.06.013
Received 9 March 2018; Received in revised form 19 June 2018; Accepted 27 June 2018
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Please cite this article as: Geurden, T., Ticks and Tick-borne Diseases (2018), https://doi.org/10.1016/j.ttbdis.2018.06.013

changes in habitat and human behavior (Randolph, 2004; Beugnet and Marié, 2009; Dantas-Torres et al., 2012). For *I. ricinus*, a latitudinal and altitudinal spread has been described, as well as increased distribution within endemic areas (Medlock et al., 2013). Similarly *D. reticulatus* is continuing to spread into novel areas (Földvári et al., 2016; Rubel et al., 2016), potentially leading to an increased reporting of canine babesiosis caused by *B. canis* (Phipps et al., 2016). Although outdoor cats are exposed to ticks and to the pathogens they transmit, the knowledge on the role of cats in the epidemiology or ecology of tick-borne pathogens is limited (Otranto et al., 2017; Pennisi et al., 2015).

The widespread occurrence and the ability of ticks to transmit important pathogens warrant regular screening of cats and dogs for tick infestation. In addition, as companion animals live in close proximity to their owners, the collection of ticks from infected animals combined with a screening for tick-borne pathogens can also provide information about the potential infection pressure for the human population (Shaw et al., 2001; Baneth, 2014; Otranto et al., 2014). Good medical practice would be to recommend routine use of appropriate anti-parasitic products with an acaricide spectrum to prevent transmission of infections (Baneth et al., 2012). The objective of the present study was to examine the presence of tick-borne pathogens in ticks collected from dogs and cats presented at their local veterinary practice in different European countries.

2. Material and methods

2.1. Tick collection

Ticks were collected from dogs and cats that were enrolled in two field patient studies to evaluate the efficacy and safety of sarolaner in the field (Becskei et al., 2016; Geurden et al., 2017). A list of the study sites with a geo-reference is provided in the supplement. Animals were enrolled during the tick season (April to August) prior to the first acaricide treatment. In the dog study, ticks were collected from dogs presented at veterinary practices in Belgium (4 clinics), France (8 clinics), Hungary (5 clinics) and Italy (5 clinics), respectively. In the cat study, ticks were collected in Germany (11 clinics), in France (8 clinics) and from 7 sites in both Hungary and Italy. The veterinary practices participating in the study were selected based on their potential to enroll tick-infested animals. All animals were presented at the veterinary practice for a variety of reasons and not specifically for symptoms related to tick infestation or tick-borne infections, and only healthy animals infested with a total count of 3 or more ticks were selected. Although the presence of tick-borne was not specifically examined in these studies, no adverse events linked to tick-borne infections were reported during the 3 month efficacy evaluation period after the collection of the ticks that were examined in this study.

Ticks were identified at species level based on their morphology (Hillyard, 1996; Estrada-Pena et al., 2004). For each individual animal, the ticks were pooled per tick species. If more than one but less than 10 ticks were collected, all of the ticks were pooled in one tick sample. If more than 10 ticks from the same tick species were collected from a dog or a cat, 10 ticks were randomly selected and pooled. All ticks were preserved in 70% ethanol.

2.2. DNA extraction from pooled tick samples and PCR detection of tick-borne pathogens

Genomic DNA was extracted from ticks using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the instruction of the kit's manual for tissue protocol. Prior to the DNA extraction ticks were removed from the 70% ethanol, dried in a Petri dish and washed in water containing washing-up liquid followed by rinsing in distilled water. Depending on the size some ticks were cut in half in a medio-sagittal direction so that the salivary glands remained intact. If fully fed, ticks were cut again into two and only the parts with salivary glands were transferred into a labelled Eppendorf tube containing 100 µl PBS. The tick parts were sliced to smaller pieces using sterilized scissors. For each tick sample, a new sterile blade was used to avoid possible contamination between tick samples. After the PCR, the PCR products were run over a 1.5% agarose gel (100 V, 40 min), stained with ethidium-bromide and visualized under ultra-violet light. Selected PCR products were purified and sequenced by Biomi Inc. (Gödöllő, Hungary). All of the sequences were compared to the NCBI Nucleotide Database.

A selection of pathogens was examined in the respective tick species, as following:

- *A.phagocytophilum* and *Borrelia* spp. in I. *ricinus* and I. *hexagonus*
- *Babesia* spp. in *D. reticulatus*
- *Hepatozoon* spp., *Babesia* spp., *Rickettsia* spp. and Anaplasma platys in R. *sanguineus* s.l.

Anaplasma phagocytophilum DNA was detected using a probe-based real-time PCR as described in Courtney et al. (2004) and specific primers targeting the *msp2* gene (77 bp). *Borrelia* spp. DNA was detected by amplification of the variable 5 S–23 S intergenic spacer region (IGS), as described by Szekeres et al. (2015). For the detection of *Babesia* spp., a conventional PCR was used to amplify a ∼500-bp fragment of the 18S rRNA gene (Casati et al., 2006). *Hepatozoon* spp. DNA was detected by amplification of a 650-bp fragment of the 18S rRNA gene (Inokuma et al., 2002).

For the detection of *Anaplasma platys* and *Rickettsia* spp., a species-specific PCR reaction was used to detect the presence of the 520-bp portion of the *Anaplasma platys p44* gene. The amplification was performed with the primers Apl_p44F3 : 5′-GCT AAG TGG AGC GGT GGC GAT GAC AG-3′ forward and Apl_p44R3: 5′- CGA TCT CCG CCG CTT TCG TAT TCT TC – 3′ reverse (Arraga-Alvarado et al., 2014), in a 25 µl final volume reaction mixture containing 5 µl DNA template, 1.0 U HotStar Taq Plus DNA Polymerase (5 U / µl) (QIAGEN®, Hilden, Germany), 2.5 µl 10x CoralLoad PCR buffer (15 mM $MgCl_2$ incl.), 0.5 µl dNTP mix (10 mM), 0.3 µl of each primer (50 u M) and 16.2 µl ddH2O. An initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 95 °C for 40 s, annealing at 62 °C for 40 s and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 7 min. DNA of sequenced *A. platys* served as positive control.

For the genus-specific detection of Spotted Fever Group Rickettsiae, a PCR was used (Regnery et al., 1991), to amplify a ∼380-bp fragment of the gltA gene with the forward primer RpCS.877p 5′-GGG GGC CTG CTC ACG GCG G-3′ and the reverse primer RpCS.1258n 5′-ATT GCA AAA AGT ACA GTG AAC A-3′. 2.5 µl of extracted DNA were added to 22.5 µl of reaction mixture containing 1.0 U HotStar Taq Plus DNA Polymerase (5 U / µl) (QIAGEN®, Hilden, Germany), 0.5 µl dNTP Mix (10 mM), 0.5 µl of each primer (50 µM), 2.5 µl of 10x Coral Load PCR buffer (15 mM $MgCl_2$ included), and 18.3 µl DW. An initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 48 °C for 30 s and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 5 min. DNA of the three targeted *Rickettsia* spp. served as positive control.

2.3. Molecular identification of Rhipicephalus sanguineus s.l

To investigate the *R. sanguineus* s.l. tick lineage, 66 tick specimens (35 collected in France and 31 collected in Italy) were selected for further genetic analysis, including at least one tick from each geographical site and host. Partial mitochondrial 16S rRNA (∼300 bp) gene sequences were generated using primers and PCR run conditions described elsewhere (Burlini et al., 2010). For phylogenetic analysis, sequences from each haplotype obtained as well as from individual or consensus sequences of the other *Rhipicephalus* spp. from a previous study (Dantas-Torres et al., 2013) were included (i.e., *R. sanguineus* s.l.: KC243835–KC243838; *R. sanguineus* sp. II-temperate lineage: KC243843–KC243847 and KY216135–KY216141; *Rhipicephalus*

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