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ORIGINAL ARTICLE

First broad-range molecular screening of tick-borne pathogens in *Ixodes* (*Pholeoixodes*) *kaiseri*, with special emphasis on piroplasms

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ABSTRACT

Recently, the occurrence of *Ixodes* (*Pholeoixodes*) *kaiseri* has been reported for the first time in several European countries, but data on the molecular analysis of this hard tick species are still lacking. Therefore, in this study DNA extracts of 28 *I. kaiseri* (collected from dogs and red foxes in Germany, Hungary and Romania) were screened with reverse line blot hybridisation (RLB), PCR and sequencing for the presence of 43 tick-borne pathogens or other members of their families from the categories of Anaplasmataceae, piroplasms, rickettsiae and borreliae. *Rickettsia helvetica* DNA was detected in one *I. kaiseri* female (from a red fox, Romania), for the first time in this tick species. Six ticks (from red foxes, Romania) contained the DNA of *Babesia vulpes*, also for the first time in the case of *I. kaiseri*. Molecular evidence of *R. helvetica* and *B. vulpes* in engorged *I. kaiseri* does not prove that this tick species is a vector of the above two pathogens, because they might have been taken up by the ticks from the blood of foxes. In addition, one *I. kaiseri* female (from a dog, Hungary) harboured *Babesia* sp. badger type-B, identified for the first time in Hungary and Central Europe (i.e. it has been reported previously from Western Europe and China). The latter finding can be explained by either the susceptibility of dogs to *Babesia* sp. badger type-B, or by transstadial survival of this piroplasm in *I. kaiseri*.

KEYWORDS

Rickettsia, *Babesia*, carnivores, red fox, RLB

The subgenus *Pholeoixodes* belongs to the most species-rich genus of hard ticks (Acari: Ixodidae: *Ixodes*). *Pholeoixodes* species are usually associated with 'pholeophilic' mammals and birds, which are named as such because they prefer to hide in cavities (implying burrow-dwelling mammals as well as terrestrial birds that nest in tree holes or burrows: Hornok et al., 2017). In the Western Palaearctic, five species of this subgenus feed on domestic and wild carnivores (mainly Canidae, Mustelidae), i.e. *Ixodes canisuga* Johnston, 1849, *I. kaiseri* Arthur, 1957, *I. crenulatus* Koch, 1844, *I. hexagonus* Leach, 1815 and *I. rugicollis* Schulze and Schlottke, 1929. Among these, *I. rugicollis* is regarded as very rare, and data on the occurrence of *I. crenulatus* in Europe appear to be either historical or uncertain (Hornok et al., 2017). On the other hand, *I. canisuga*, *I. hexagonus* and *I. kaiseri* commonly infest dogs and foxes in many

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European countries (Hornok et al., 2017; Sándor, 2017a,b). Considering these three species, several molecular studies have been conducted to screen pathogens in *I. canisuga* and *I. hexagonus* (reviewed in Sándor, 2017a,b; Hornok et al., 2018a). However, reports on PCR-based screening of pathogens in *I. kaiseri* are missing (Estrada-Peña, 2017), also taking into account that in a report from Poland ticks resembling *I. kaiseri* were later shown to be *I. canisuga* (Wodecka et al., 2016; in GenBank: KF471772).

In this study, whole body DNA extracts of 28 *I. kaiseri* specimens were used (Table 1). These ticks (originally collected from four red foxes in Germany, from eight dogs in Hungary, and from one dog and 15 red foxes in Romania) were molecularly identified following morphological comparison to type specimens (Hornok et al., 2017). In order to screen these samples for a broad range of tick-borne pathogens, reverse line blot hybridisation (RLB) was performed (Kirstein et al., 1997), modified as previously published (Schötta et al., 2017). The oligonucleotides included group-level (catch-all) probes for *Anaplasma*/*Ehrlichia* spp., *Theileria*/*Babesia* spp., *Borrelia burgdorferi* sensu lato and *Rickettsia* spp. The species-specific probes targeted eight species from Anaplasmataceae, 17 species of piroplasms, eight species of borreliae and ten *Rickettsia* species (Schötta et al., 2017).

In addition, to confirm RLB results, the PCR products were sequenced. The *R. helvetica*-positive sample was tested by a PCR, amplifying an approximately 480-bp-long fragment of the 17 kDa surface antigen gene of *Rickettsia* spp., with the primers 17kd1 (5′-GCT CTT GCA ACT TCT ATG TT-3′) and 17 kd2 (5′-CAT TGT TCG TCA GGT TGG CG-3′) as described (Hornok et al., 2018b). Piroplasm-positive samples were further analysed with a PCR, amplifying a ~500 bp region of the 18S rRNA gene, with the primers BJ1 (forward: 5′-GTC TTG TAA TTG GAA TGA TGG-3′) and BN2 (reverse: 5′-TAG TTT ATG GTT AGG ACT ACG-3′). The method was modified from Casati et al. (2006) as reported in Hornok et al. (2016). Purification and sequencing from the latter two PCRs were performed by Biomi Inc. (Gödöllő, Hungary). The new sequences were compared to GenBank sequences by the nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). Representative sequences were submitted to GenBank (accession numbers: MK733576, MK733577 and MK733578-9 for the gltA, 17 kDa and 18S rRNA gene sequences, respectively).

One *I. kaiseri* female (collected from a red fox in Romania: Table 1) was positive for *Rickettsia helvetica* in the RLB. The short gltA sequence from this sample (GenBank: MK733576) had 100% (283/283 bp) identity only to *R. helvetica* sequences deposited in GenBank (e.g. detected in *I. persulcatus*, Russia: KU310588). The amplified part of the 17 kDa antigen gene (GenBank: MK733577) confirmed this result, because it showed 100% (388/388 bp) identity with isolates of *R. helvetica* (e.g. detected in *I. ricinus*, Italy: KY346828).

This finding should be interpreted with caution, because it is not known (1) if *R. helvetica* was present in *I. kaiseri* prior to its engorgement (i.e. transmitted transovarially and/or transstadially from a previous generation or stage), or (2) if these bacteria have been taken up by the tick from the blood of its host. In support of this second possibility, red foxes are known to be bacteraemic with *R. helvetica*, although rarely (Hofmann-Lehmann et al., 2016). On the other hand, the first explanation is also plausible, considering that *R. helvetica* was isolated from several *Ixodes* species, some outside the species complex of its known vector, *I. ricinus* (Parola et al., 2013).

Six *I. kaiseri* specimens (all collected from red foxes in Romania: Table 1) were positive for *Babesia vulpes* in the RLB. The 18S rRNA sequences from all six samples (GenBank: MK733578) were 100% (454/454 bp) identical with each other and to those deposited in GenBank from several countries (including Romania, from golden jackal: KX712130). *Babesia vulpes* is known to occur in red foxes in Romania (Daskalaki et al., 2018), therefore molecular evidence of its presence in engorged *I. kaiseri* does not prove vector competence of the latter (i.e. this piroplasm might have been taken up by the ticks from the blood of foxes). The most likely vector of *B. vulpes* is *I. hexagonus* (Camacho et al., 2003), but it was also shown to be present in *I. canisuga* (Najm et al., 2014) and here for the first time in *I. kaiseri*. This means that all three *Pholeoixodes* species, which infest red foxes, may acquire *B. vulpes* and should be evaluated further (i.e. compared) in their potential vector role to transmit this piroplasm.

Table 1. Collection data and results of molecular analyses of *Ixodes kaiseri* samples used in this study.

Country	Locality	Hosts of origin	Tick developmental stage or adult sex (number)	PCR positive / all tested (result of sequencing)	GenBank accession numbers (gene)
Germany	Thuringia	Red foxes	Females (4×)	0/4	–
Hungary	Budapest	Dogs	Females (8×)	1/8 (*Babesia* sp. badger type-B)	MK733579 (18S rRNA)
Romania	Iazurile	Dog	Female (1×)	0/1	–
	Cefa	Red fox	Nymph (1×)	1/1 (*Babesia vulpes*)	MK733578 (18S rRNA)
	Sălard	Red fox	Female (1×)	0/1	–
	Popeşti	Red foxes	Females (2×), nymph (1×)	1/3 (*Babesia vulpes*)	MK733578 (18S rRNA)
	Sânpetru	Red foxes	Females (4×), nymph (4×)	2/8 (*Babesia vulpes*)* and 1/8 (*Rickettsia helvetica*)*	MK733578 (18S rRNA) and MK733576 (gltA), MK733577 (17 kDa antigen)
	Ilia	Red foxes	Female (1×), nymph (1×)	2/2 (*Babesia vulpes*)	MK733578 (18S rRNA)

The asterisk marks the simultaneous presence of DNA from two pathogens in the same tick

In addition, one *I. kaiseri* female (removed from a dog in Hungary: Table 1) gave a positive signal with the *Babesia* catch-all probe but was negative for all *Babesia* species included in the test with species-specific probes. The 18S rRNA sequence from this sample (GenBank: MK733579) was 99.8–100% (439–440/440 bp) identical only to *Babesia* sp. badger type-B, represented by four sequences in GenBank (MG799846 from China and KT223485, KX528554–KX528555 from the UK, all from European badgers) (Barandika et al., 2016; Bartley et al., 2017). This appears to be the most significant finding of the present study. In a geographical context, to the best of our knowledge, this is the first report of this piroplasm from Central Europe, where hitherto only *Babesia* sp. badger type-A has been detected (Hornok et al., 2018a). Moreover, molecular identification of *Babesia* sp. badger type-B in an *I. kaiseri* adult from a dog has two likely explanations, both significant enough to be further evaluated. The first possibility is that the relevant tick ingested this piroplasm from the blood of its canine host. If so, this would be the first indication that dogs are susceptible to this badger-associated piroplasm, as suggested for the closely related species, *Babesia* sp. badger type-A in a recent study (Hornok et al., 2018a). It is relevant to note that badgers (although rarely) occur within Budapest (data not shown), and urban badger populations were reported to increase in other cities of Central Europe (Geiger et al., 2018).

Second, if the female tick carried *Babesia* sp. badger type-B prior to its blood meal, that would imply transstadial survival and potential transmission of this piroplasm by *I. kaiseri*. This is especially important to consider in light of the fact that two further piroplasms were shown to associate significantly with the other two *Pholeoixodes* tick species commonly infesting burrow-dwelling carnivores in Europe, i.e. *B. vulpes* with *I. hexagonus* (Camacho et al., 2003) and *Babesia* sp. badger type-A with *I. canisuga* (Hornok et al., 2018a). Thus, *I. kaiseri* should be included in future transmission experiments aimed at assessing the vector competence of *Pholeoixodes* species in the transmission of *Babesia* sp. badger type-B.

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