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Molecular investigation of hard ticks (Acari: Ixodidae) and fleas (Siphonaptera: Pulicidae) as potential vectors of rickettsial and mycoplasmal agents

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ABSTRACT

The aim of the present study was twofold. First, in general, to reveal new aspects of the potential vector role of ixodid ticks and fleas by screening large numbers of specimens with recently developed molecular biological methods. Second, to evaluate the occurrence of vector-borne infectious agents in a geographical context. Altogether 3442 unfed hard ticks (*Ixodes ricinus*, *Dermacentor marginatus*, *D. reticulatus*, *Haemaphysalis inermis*, *H. concinna*, *H. punctata*) and 939 fleas of cats and dogs (*Ctenocephalides felis*, *C. canis*, *Pulex irritans*) were collected in Hungary. DNA was extracted and analyzed in pools for representatives of the orders Rickettsiales and Mycoplasmatales.

H. inermis was newly identified as the most important potential vector for *Rickettsia helvetica* in the study region. A novel *Rickettsia* genotype (designated '*Candidatus* R. hungarica') was also detected in the same tick species, with a maximum of 95.8% *gltA* gene sequence identity to known rickettsiae. In addition, *P. irritans* tested positive for *Rickettsia* sp. RF2125, which has not been previously described in Europe. The human pathogen *R. felis* and the feline pathogen '*Candidatus* Mycoplasma turicensis' were shown for the first time to occur in Central-Eastern Europe. Further novel findings include the presence of *Spiroplasma* spp. in *D. marginatus* and fleas. In conclusion, this molecular study extends the geographic range and vector spectrum of several arthropod-borne agents, some of which have zoonotic potential.

1. Introduction

Blood-sucking arthropod vectors, exemplified by ticks and fleas, are able to transmit infectious agents between animals and/or humans. Most of the pathogenic bacteria belonging to the order Rickettsiales are vector-borne (Dumler et al., 2001), and this transmission route has also been suggested for several representatives of the order Mycoplasmatales (Neimark et al., 2001).

Rickettsia spp. (family Rickettsiaceae, order Rickettsiales) are Gram-negative, obligate intracellular bacteria (Dumler et al., 2001). Although they have been long known for their public health importance, recently an emerging veterinary significance of various species was also recognized (Hechemy et al., 2006). Similarly, *Ehrlichia* spp. (family Anaplasmataceae, order Rickettsiales) include both human and animal pathogens (Dumler et al., 2001).

Haemotropic mycoplasmas (also known as haemoplasmas; order Mycoplasmatales), formerly assigned to the

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Table 1
Pooling criteria for ticks and fleas.

	Total number	Number of adults per pool	Number of immature stages per pool[a]	Total number of pools
Ticks	3,442			
Ixodes ricinus	2,116	20 ♂, 10 ♀	30 n, 24 l	41 ♂, 94 ♀, 9 n, 1 l
Dermacentor marginatus	369	5 ♂, 5 ♀	–	23 ♂, 34 ♀
D. reticulatus	361	5 ♂, 5 ♀	–	25 ♂, 46 ♀
Haemaphysalis inermis	315	5 ♂, 5 ♀	–	24 ♂, 40 ♀
H. concinna	259	3 ♂, 3 ♀	10 n, 52 l	29 ♂, 16 ♀, 7 n, 1 l
H. punctata	22	3 ♂, 3 ♀	1 n	5 ♂, 3 ♀, 1 n
Fleas	939			
Ctenocephalides felis	393	1–11	–	187
C. canis	526	1–12	–	195
Pulex irritans	20	1–4	–	15

[a] *Abbreviations*: n, nymph; l, larva.

genera *Haemobartonella* and *Eperythrozoon* (Neimark et al., 2001), attach to red blood cells. This may lead to haemolytic anaemia in infected domestic and wild animals. Vectors of haemoplasmas may include fleas, hard ticks, and mosquitoes (Neimark et al., 2001).

Spiroplasma spp. are helical mycoplasmas. Although they have been implicated in spongiform encephalopathies (Bastian et al., 2007), unambiguous information on their exact role in diseases is still lacking. Various categories of insects and arachnids, including certain hard ticks, such as *Ixodes ricinus*, have been shown to harbor these bacteria (Carle et al., 1995).

In Hungary *Rickettsia slovaca*-like organisms have been found in questing (unfed) *Dermacentor* spp. (Rehácek et al., 1979). More recently engorged ticks were investigated: *R. helvetica* and *R. monacensis* have been detected in *I. ricinus*, *R. raoultii* (formerly *Rickettsia* sp. RpA4) in *Dermacentor reticulatus*, and a distinct rickettsia genotype in *Haemaphysalis concinna* (Sréter-Lancz et al., 2006). However, no other tick species – particularly not questing ones – or flea species have been evaluated. Similarly, no data have been published to date on the occurrence of *Ehrlichia canis*, haemotropic *Mycoplasma*, or *Spiroplasma* spp. in ticks or fleas of this region in Europe. Therefore, the present study was undertaken to obtain relevant and updated information on these important vector-borne agents.

2. Materials and methods

2.1. Origin of the samples

Between March and July 2007, a total of 3442 hard ticks (Acari: Ixodidae) were collected from vegetation at 37 locations in Hungary using the cloth-dragging and flagging method. The species, stage, and sex of the ticks were determined by microscopic examination. Subsequently, ticks were allotted into pools (Table 1) taking into account their size, i.e. for optimal DNA extraction. The last pool of each species contained the remainder of specimens.

In addition, 939 fleas (Siphonaptera: Pulicidae) were collected (420 from cats and 519 from dogs) at 14 small animal clinics in various parts of the country. All fleas of one species removed from one animal were pooled (Table 1). *Ctenocephalides felis* were obtained from cats, *C. canis* from cats and dogs, and *Pulex irritans* from dogs. Both tick and flea samples were stored in 70% ethanol until molecular biological analysis.

2.2. DNA extraction

Pools of ticks and fleas were air-dried, washed twice in phosphate-buffered saline without $MgCl_2$ or $CaCl_2$ (DPBS; Invitrogen, Basel, Switzerland) and minced with scissors in 100 μl DPBS at the bottom of 2 ml Eppendorf tubes. Between the preparations of pools, the scissors were decontaminated by washing in tap water, burning, and cooling in distilled water.

From all tick pools and from those of *C. canis* and *P. irritans*, DNA was extracted using the MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics, Rotkreuz, Switzerland). Briefly, 150 μl of DPBS and 375 μl of lysis buffer containing guanidinium thiocyanate and Triton X-100 were added to each pool, together with a 5 mm stainless steel bead (Schieritz and Hauenstein AG, Arlesheim, Switzerland). The samples were homogenized in a Mixer Mill device (Retsch GmbH, Haan, Germany) for 2 min at 30,000 Hz. After a short centrifugation at 5000 × *g*, 500 μl of the supernatant from each sample was loaded onto the MagNA Pure LC Instrument (Roche Diagnostics). Two negative extraction controls containing only DPBS were included per 30 samples in order to monitor for cross-contamination. DNA from the 187 pools of *C. felis* was extracted using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany). In both procedures the elution volume was 100 μl.

The presence of amplifiable DNA was confirmed for each pool using a real-time PCR assay to detect the 18S rRNA gene (Applied Biosystems, Rotkreuz, Switzerland). The concentration of amplifiable DNA was determined as sufficient if the threshold cycle (C_t) value was less than 25. Samples were stored at −20 °C until analysis.

2.3. Real-time PCRs

All samples were first screened with real-time PCR. Cycling conditions were: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Ninety-three samples (including three negative extraction controls) were analyzed in one assay, together with two – external and internal – negative and one