Szent István University
Postgraduate School of Veterinary Science

Studies of ticks (Acari: Ixodidae) and tick-borne pathogens of dogs in Hungary

PhD dissertation

By
Gábor Földvári

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Szent István University
Faculty of Veterinary Science
Postgraduate School of Veterinary Science
Budapest, Hungary

Supervisor:

…….
Róbert Farkas, DVM, PhD
Dep. of Parasitology and Zoology, Fac. Vet. Sci., SzIU

Co-supervisors:

Prof. Miklós Rusvai, DVM, PhD
Dep. of Pathology and Forensic Vet. Medicine, Fac. Vet. Sci., SzIU

Prof. Károly Vörös, DVM, PhD, DSc
Dep. and Clinic of Internal Medicine, Fac. Vet. Sci., SzIU

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Gábor Földvári

CONTENTS

1. SUMMARY

In Europe, the number of reports on canine tick-borne diseases has increased in the past few years. In Hungary, we have had very limited information concerning tick infestation and tick-borne pathogens of dogs. For these reasons, we started to study the tick species and tick-borne pathogens infecting dogs in our country.

Based on morphological studies, a figured practical identification key has been designed for the sixteen hard tick species which have been found on dogs in Europe. The simplicity of this key can help veterinarians and zoologists in tick identification.

In 29 veterinary clinics from six districts of Budapest and 13 counties, 1779 tick specimens were collected from 606 dogs. Most hosts were usually infested with a single female and very few of them had many ticks. The most preferred sites of tick attachment in decreasing order were head, neck and legs. *Ixodes ricinus* and *Dermacentor reticulatus* were the most common species. *Ixodes canisuga, Haemaphysalis concinna, Ixodes hexagonus, Ixodes acuminatus* and *Dermacentor marginatus* were also found. New data have been provided about the geographical distribution of *Dermacentor reticulatus*, because the specimens of this species were collected in north-eastern and south-eastern parts of the country too where they had not been found before. Field collections in 31 locations provided new data on the geographical and seasonal occurrence of *I. ricinus, D. reticulatus* and other tick species as well.

The occurrence of small canine piroplasms in two dogs was described for the first time in Hungary. These were autochtonous infestations but we need further investigations to know the species, occurrence, vector and origin of this pathogen. The subspecies *Babesia canis canis* was identified to be the causative agent of babesiosis caused by large *Babesia* sp. in dogs using molecular biological methods. It was also proven with molecular methods that the geographical distribution of canine babesiosis is larger in the country than it has been previously known. *Babesia* DNA was detected in free-living and engorged *D. reticulatus* females for the first time in the country. Presence of *B. canis canis* in engorged *D. reticulatus* specimens removed from dogs was also demonstrated with molecular methods.

Molecular evidence was found for the presence of *Borrelia* sp. in free-living and engorged *I. ricinus* females for the first time in Hungary. Three species, *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* were identified with sequence analysis which are pathogenic to both dogs and humans.

2. INTRODUCTION

Biological complexity of pathogen-tick-host systems is exceptional. More than 3500 years ago, both the Ancient Egyptians and the Ancient Greeks were already aware of ticks and their medical importance (Varma, 1993). Smith and Kilbourne (1893) completed first landmark research proving that Texas fever (caused by *Babesia bigemina*) was spread from one cow to another by hard ticks (*Boophilus annulatus*), just as many cattlemen had suspected. Their discovery spurred the search for vectors of malaria-causing *Plasmodium* and other pathogens worldwide (Pratt and Littig, 1962). Since this first proof of hard tick's ability to transmit disease agents, scientific attention has continuously been raising. Considering the human and veterinary health importance of ixodid ticks, the scope for exciting novel discoveries is immense. Only during the last 30 years, a considerable increase occurred in the number of publications containing the word "tick" within the ISI Web of Science® database (Fig. 1). Ticks are considered second only to mosquitoes as vectors of human infectious disease agents worldwide but first in Eurasia and North America (Estrada-Peňa and Jongejan, 1999). In Europe during the last two decades there has arisen an increased awareness of ticks and the pathogens they carry (Hillyard, 1996).

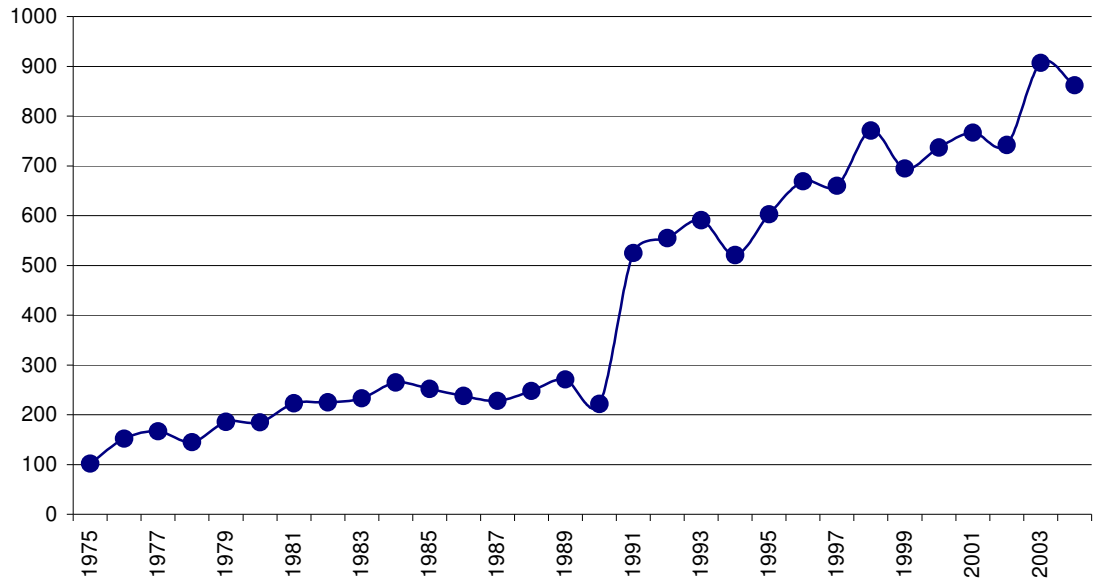

Figure 1. Number of publications containing the word "tick" between 1975-2004 within the ISI Web of Science® database.

Hard ticks (Acari: Ixodidae) are obligate hematophaguos ectoparasites of a wide variety of terrestrial vertebrates including domestic dogs. Originally evolved as parasites of wild animals, only

a relative minority of the approximately 650 hard tick species, generally those with wide host range can transmit diseases to domesticated animals (Shaw et al., 2001). Because of the wide range of transmitted pathogens, ticks are of considerable medical and veterinary interest worldwide. In Europe, the number of reports of canine tick-borne diseases has increased in the past few years (Shaw et al., 2001; Chandoga et al., 2002; Camacho et al., 2003; Criado-Fornelio et al., 2003a,b). Emerging tick transmitted canine diseases like babesiosis, anaplasmosis, ehrlichiosis, rickettsiosis, mycoplasmosis, hepatozoonosis and borreliosis have drawn both public and scientific attention to these arthropods (Beugnet, 2002; Kenny et al, 2004a).

Increased mobility of pets and the ability of ticks to find niches in new climatic conditions have resulted in rapid extension of the zoogeographical ranges for many tick species (Glaser and Gothe, 1998; Shaw et al., 2001). The increasing number of ticks has also been associated with growing accessibility of natural environments and an increase in the population of wild host species (deer, small mammals and foxes) that now have a closer association with human activity. For example, *Ixodes ricinus* has extended its range in Sweden to more northern and western areas since the 1980`s (Talleklint and Jaenson, 1998). Vector of several canine pathogens, *Rhipicephalus sanguineus*, has also a good adaptive ability and is likely to inhabit new areas throughout Europe (Gothe, 1968; Gothe and Hamel, 1973; Fox and Sykes, 1985; Gothe, 1999). Recently, it has been reported that importation of *Dermacentor reticulatus* into new regions poses higher risk for canine babesiosis (Zahler and Gothe, 1997 Zahler et al., 2000a).

The importance of ticks is also highlighted by the rapid development of molecular biological methods enabling easy screening of blood samples and ticks for disease agents (Sparagano et al., 1999; Criado-Fornelio et al., 2003b; Monis et al., 2005). There has been an increased awareness of dogs' ticks because some of them can be dangerous also to humans transmitting zoonotic diseases (Shaw et al., 2001; Beugnet, 2002).

2.1. Hard tick infestation of dogs

Studies examining the hard tick infestation of dogs have been published from the mid 1980s in Europe. Liebisch et al. (1984) recorded the following seven tick species collected from 1624 dogs in Germany (in decreasing order of occurrence): *I. ricinus, Ixodes hexagonus, D. reticulatus, R. sanguineus, Ixodes canisuga, Dermacentor marginatus* and *Haemaphysalis concinna.* Beichel et al. (1996) reported the occurrence of only two species, *I. ricinus* and *I. hexagonus* on 48 infested animals in Germany. Grandes (1986) found *R. sanguineus, Rhipicephalus turanicus, Rhipicephalus pusillus, Rhipicephalus bursa, I. ricinus* and *Hyalomma marginatum marginatum* on 179 dogs in Salamanca region, Spain. Papadopoulos et al. (1996) examined ticks from 70 dogs in Greece and

found *R. sanguineus, R. turanicus, I. ricinus, R. bursa, I. hexagonus* and *Haemaphysalis punctata* in decreasing order of frequency. Papazahariadou et al. (2003) found only two species, *R. sanguineus* and *R. turanicus* on 249 tick-infested animals in northern Greece. Ogden et al. (2000) identified *I. ricinus, I. hexagonus, I. canisuga, H. punctata* and *D. reticulatus* collected from 213 dogs in Great Britain and Ireland. Concerning occurrence, veterinary and zoonotic importance, *Ixodes ricinus, Rhipicephalus sanguineus* and *Dermacentor reticulatus* (Fig. 2-4) are the most important species infesting dogs in Europe (Shaw et al., 2001; Beugnet, 2002).

Figure 2. *Ixodes ricinus* male and unengorged female.

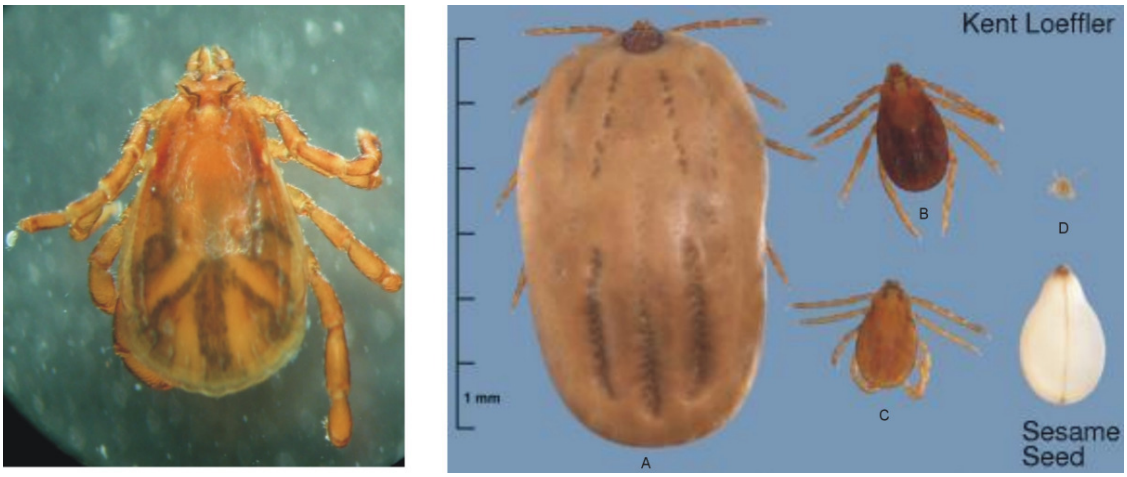

Figure 3. *Rhipicephalus sanguineus* male, fully engorged female (A), unfed female (B), male (C) and larva (D). Scale is valid only for the right hand picture modified from http://www.entomology.cornell.edu/MedEnt/TickBioFS/TickBioFS.html.

Figure 4. *Dermacentor reticulatus* male and unengorged female.

In Hungary the first comprehensive studies on the occurrence of ixodid tick species were made several decades ago (Kotlán, 1919,1921). Although there is a limited number of publications, in some of them the use of species names (especially in case of *D. reticulatus*) produced misunderstandings in the later papers. Kotlán (1919) for example used the name *D. reticulatus* correctly applying the principle of priority which is commonly accepted in zoological nomenclature. Janisch (1959) and Babos (1965) however, considered the name *Dermacentor pictus* to be valid name for the species with the same morphological characters. Further complications aroused when Kotlán and Kobulej (1972) and Janisch (1986) considered *D. reticulatus* and *D. marginatus* to be synonyms, however, they are morphologically clearly distinct (Arthur, 1960).

Janisch (1959) collected approximately 15000 tick specimens from mammals, birds and from field. He reported that *I. ricinus* and *D. marginatus* were the most common species in the country. He had knowledge on the occurrence of *D. reticulatus* (syn. *D. pictus*) merely from Fertőd and Tolna which he explained with importation. Babos (1965) included 33 species of five genera into his identification key of hard tick species of Hungary. However, on the basis of current valid species names (Camicas et al., 1998; Horak et al., 2002), these are in fact 24 valid species, 19 of which were registered to occur in the country. Seven of these species, *I. ricinus, I. canisuga, H. concinna, Haemaphysalis inermis, Haemaphysalis parva* (syn. *Haemaphysalis otophila*), *D. reticulatus* (syn. *D. pictus*), and *D. marginatus* were mentioned to infest dogs in Hungary. Until recently, there has been no information about the temporal and spatial distribution of hard tick species infesting dogs. Farkas and Földvári (2001) examined 160 tick specimens collected from 100 dogs. Four species were found of which *I. ricinus* and *D. reticulatus* were the most common. One specimen of *D. marginatus* and *I. hexagonus* also occurred. Significant association was found between the presence of clinical signs of canine babesiosis and the infestation of these animals with *D. reticulatus* (γ=0.53, Px4<< 0.001). The latter species occurred in a greater geographic range than Babos (1965) and Janisch (1959, 1986) previously described.

There have been no other data published on the tick species of dogs, however in a recent study, *I. ricinus, H. concinna, D. reticulatus* and *I. canisuga* were found on red foxes in Hungary (Sréter et al., 2003). These carnivores can be considered as relevant to the natural maintenance of tick species that are able to feed on dogs, because the number of foxes is still high (>60000) in the country (Csányi, 2005).

D. reticulatus has been found to be vector of *Babesia canis*, a tick-borne pathogen of dogs common in Hungary (Janisch, 1986). Because canine babesiosis is a severe and frequent disease in the country (Horváth and Papp, 1996; Csikós et al., 2001), it is crucial to study the geographical and seasonal distribution of this tick species in particular. Previous data on tick infestation of dogs (Farkas and Földvári, 2001), and foxes (Sréter et al., 2005) suggest that the spatial distribution of *D.*

reticulatus has expanded since the 1950s. According to Meyer-König et al. (2001), this species has extended its distribution from the 31st and 40th northern parallel to the 60th northern parallel. One explanation for this is the adult's marked ecological plasticity which is also reflected by its occurrence in a variety of ecological zones. Another reason is the nidicolous life of larvae and nymphs which are active during summer only and are particularly protected against extremely unfavourable climatic conditions in the habitats of their hosts, which are burrow-dwelling and ground-living small mammals (Zahler, 1994).

The risk of occurrence of non-indigenous tick species and new tick-borne pathogens (Kálmán et al., 2003; Farkas et al., 2004; Sréter et al., 2004; Sréter-Lancz et al., 2005) has been increasing in Hungary. This may be associated with increased awareness and improved diagnostic methods but also because the number of travelling dogs (with their ticks) has been increasing (Glaser and Gothe, 1998). That is why it is important to monitor both the autochthonous and the imported tick species occurring on dogs and in the field.

Accurate identification is the essential first stage in any study involving ticks. In the case of ticks infesting dogs, it would also be important in the diagnosis of a disease because different species can transmit different pathogens. Morphological features of the adult stages of ticks have traditionally provided the main criteria for distinguishing species (Nuttall and Warburton, 1911, 1915; Arthur, 1960; Babos, 1964; Hillyard, 1996; Estrada-Peňa et al., 2004). However, morphological identification can present difficulties. For example, the large number of species in the keys that do not feed on dogs can be misleading, because most of the identification keys are designed for a particular geographical region and not restricted to host species. For this reason, a non-specialist who tries to identify a tick from a dog has to distinguish it from a number of species that are not feeding on this host. In addition, the tick's capitulum, which is usually essential for species identification, may become damaged during removal. Furthermore, for nymphal and especially for larval stage, traditional morphological identification is often ambiguous. Such difficulties have made it necessary to find methods of molecular biological identification for ixodid ticks infesting dogs. Hitherto, there have been only a few attempts to discriminate between hard tick species on the basis of their DNA sequences. However, diagnostic methods using restriction enzyme analysis of the second internal transcribed spacer (ITS-2) in the nuclear ribosomal gene have been developed for *D. reticulatus* by Zahler et al. (1995) and for 17 *Ixodes* species of the United States by Poucher et al. (1999). The usefulness of molecular methods is more restricted to phylogenetic analyses and identification of immature or damaged specimens. Morphological identification seems to be the easier and more practical method for identifying the adults which are most commonly found on dogs. A morphological key for ticks of domestic animals in the

Mediterranean region has been published recently by Estrada-Peňa et al. (2004), which includes some but not all dog-infesting species. We have, however, no information on a practical identification key which enables the identification of hard tick species that infest dogs in Europe.

2.2. Tick-borne pathogens of dogs

As hematophaguos arthropods, ticks are well designed to transmit disease agents such as viruses, bacteria, rickettsiae, protozoa, fungi and nematodes (Hillyard, 1996). Transmission of tick-borne pathogens occurs not only from tick to host but both transovarially (i.e. the eggs acquire infection from the egg-laying female) and transstadially (i.e. both larvae and nymphs are able to transmit the pathogen to the next developmental stage) (Beugnet, 2002).

Attaching of ticks firmly to their hosts, facilitates the effective blood feeding, the transmission of pathogens but also the spread of both ticks and microorganisms to different geographical habitats via migrating animals or travelling pets (Kenny et al., 2004b). Although restricted by host movement and climatic factors, many tick species and their pathogens extended their zoogeographical ranges due to the increased mobility of pets (Glaser and Gothe, 1998; Shaw et al., 2001). For example between 1995 and 1998, 36% of cases of monocytic ehrlichiosis reported in Germany occurred in dogs that had travelled for short periods to the Mediterranean area. During the same period, both *Ehrlichia canis* infection and infestation with *R. sanguineus*, the vector species traditionally found in Southern Europe, were detected in dogs that have never left Germany (Gothe, 1999).

Tick-borne encephalitis virus (TBEV) (Flaviviridae) is primary pathogenic to humans but also infects dogs (Shaw et al., 2001). Seroprevalence of the virus has been found to be higher in dogs because they come into contact with the infected vector ticks (mainly *I. ricinus*) more frequently than humans. However, the risk for developing clinical tick-borne encephalitis (TBE) in tick-infested dogs in an endemic area seems to be rather small for unknown reasons. Altogether less than twenty clinical TBE infections of dogs have been reported in Austria, Germany, Sweden and Switzerland (Beugnet, 2002).

Ehrlichia canis is an obligate intracellular Gram negative bacterium infecting white blood cells. It is transmitted by *R. sanguineus* in Europe and is the causative agent of canine monocytic ehrlichiosis which occurs worldwide and can cause serious symptoms in dogs (Beugnet, 2002).

Anaplasma phagocytophilum (formerly *E. phagocytophila,* human granulocytic ehrlichiosis agent) is also an obligate intracellular Gram negative bacterium infecting white blood cells. It can be transmitted by *I. ricinus* in Europe. *A. phagocytophilum* is the causative agent of canine anaplasmosis (granulocytic ehrlichiosis) which can produce a wide spectrum of clinical

manifestations (Beugnet, 2002).

Hepatozoon canis is an apicomplexan protozoon. Its transmission differs from other tick-borne pathogens because infection of the dog takes place by ingestion of a tick containing the parasite. Its vector species is *R. sanguineus*. Clinical signs can vary between apparently asymptomatic to severe and life-threatening disease. Infections are reported from Spain, Italy and southern France (Beugnet, 2002).

Beyond these and the two major tick-transmitted infections in Europe (babesiosis and borreliosis) there are some other tick-borne diseases like mycoplasmosis (earlier haemobartonellosis), bartonellosis, tularaemia and, rarely, louping ill (Shaw et al., 2001, Kenny et al., 2004a) as well. Several of the tick-borne infections that affect dogs can cause serious disease in humans, notably borreliosis, anaplasmosis (ehrlichiosis), Mediterranean spotted fever and tick-borne encephalitis. However, the potential zoonotic threat posed by dogs is strongly influenced by the natural cycle of the specific agent with which the dog is infected. Shaw et al. (2001) described three epidemiological scenarios. First, if the transmission of an infectious agent involves ticks with a broad host range (such as *I. ricinus*), dogs can act directly as sentinels for infection of humans. Second, by acting as natural hosts for certain nidicolous (endophilic, i.e. its non feeding stages live in the nest of their hosts) ticks (such as *R. sanguineus* and *I. canisuga*), dogs significantly increase contact between these species and humans, thereby increasing the risk of transmission (Mumcuoglu et al., 1993). Finally, there is a limited risk of transmission by exposure to infected tick-contents following damage to ticks during grooming of infested animals. This has been reported for *H. canis* (Beugnet, 2002).

2.2.1. Piroplasms

Babesia (Apicomplexa: Piroplasmida) species are tick-transmitted parasites infecting a wide range of wild and domestic vertebrate hosts (Kuttler, 1988). Traditionally, identification of species has been based on host specificity and morphology of the intraerythrocytic piroplasms. Based on these, canine babesiae have been originally recognised to belong to two distinct species, the large pyriform (4-5 µm) *Babesia canis* (Fig. 5) and the small usually pleomorph (1-2.5 µm) *Babesia gibsoni* (Fig. 6).

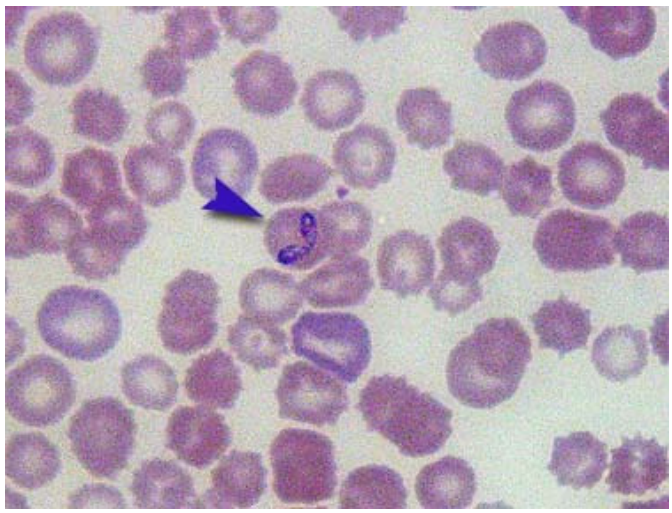

Figure 5. *Babesia canis* in a Giemsa-stained thin blood smear.

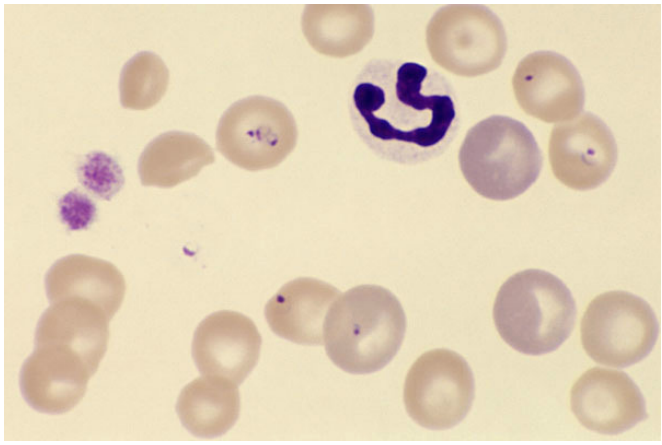

Figure 6. *Babesia gibsoni* in a Giemsa-stained thin blood smear (from http://www.yamagiku.co.jp/pathology/index.htm).

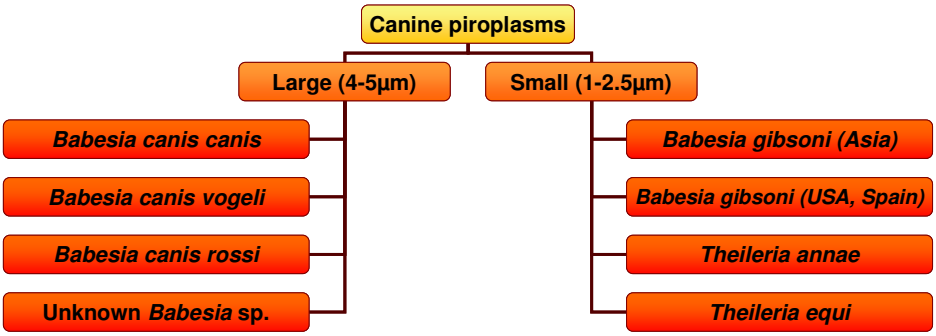

Figure 7. Piroplasms that have been described in dogs.

On the basis of differences in geographical distribution, vector specificity and antigenic properties (Uilenberg et al., 1989; Hauschild et al., 1995), *B. canis* has been subdivided into three subspecies, namely *B. canis canis* transmitted by *D. reticulatus* and *R. sanguineus* in Europe, *B. canis vogeli* transmitted by *R. sanguineus* in tropical and subtropical regions and *B. canis rossi* transmitted by *Haemaphysalis leachi* in South Africa (Fig. 7). These subspecies also differ from each other in pathogenicity. *B. canis rossi* causes a frequently fatal infection in domestic dogs, even after treatment; *B. canis vogeli* causes a moderate often clinically unapparent infection; and *B. canis canis* infections result in a more variable pathogenicity intermediate between *B. canis rossi* and B. *canis vogeli* (Uilenberg et al., 1989). Genetic differences between these subspecies have also been proved by Zahler et al. (1998) and Carret et al. (1999). Some authors (Zahler et al., 2004) suggest and some others (Passos et al., 2005) even use the species level for these three subspecies. Baneth et al. (2004) described a fourth subspecies with unknown vector named *B. canis presentii* which has been detected in cats. Recently, a novel large *Babesia* sp. has been detected in an infected dog from North America by Birkenheuer et al. (2004) which either represents a new *Babesia* sp. or is one of the nearly 100 *Babesia* spp. described for which no genetic data have been reported (Fig 8).

Canine babesiosis caused by *B. canis* has been reported in several European countries, particularly in the Mediterranean region, where *R. sanguineus* and *D. reticulatus* are its vectors. Cases of autochtonous large *Babesia* infections have been reported from Belgium, Croatia, France, Germany, Hungary, Italy, Poland, Slovenia, Spain and the Netherlands (Losson et al., 1999; Beugnet, 2002; Cacció et al., 2002; Duh et al., 2004). Recent studies using molecular methods showed that in France, Slovenia and Spain where these vector species coexist, both *B. canis canis* and *B. canis vogeli* could be detected (Cacciò et al., 2002; Criado-Fornelio et al., 2003b; Duh et al., 2004). As a contrast, in Northern Poland where neither *D. reticulatus* nor *R. sanguineus* occur, no *Babesia* sp. has been detected scanning 192 canine blood samples by PCR (Skotorczak et al., 2004).

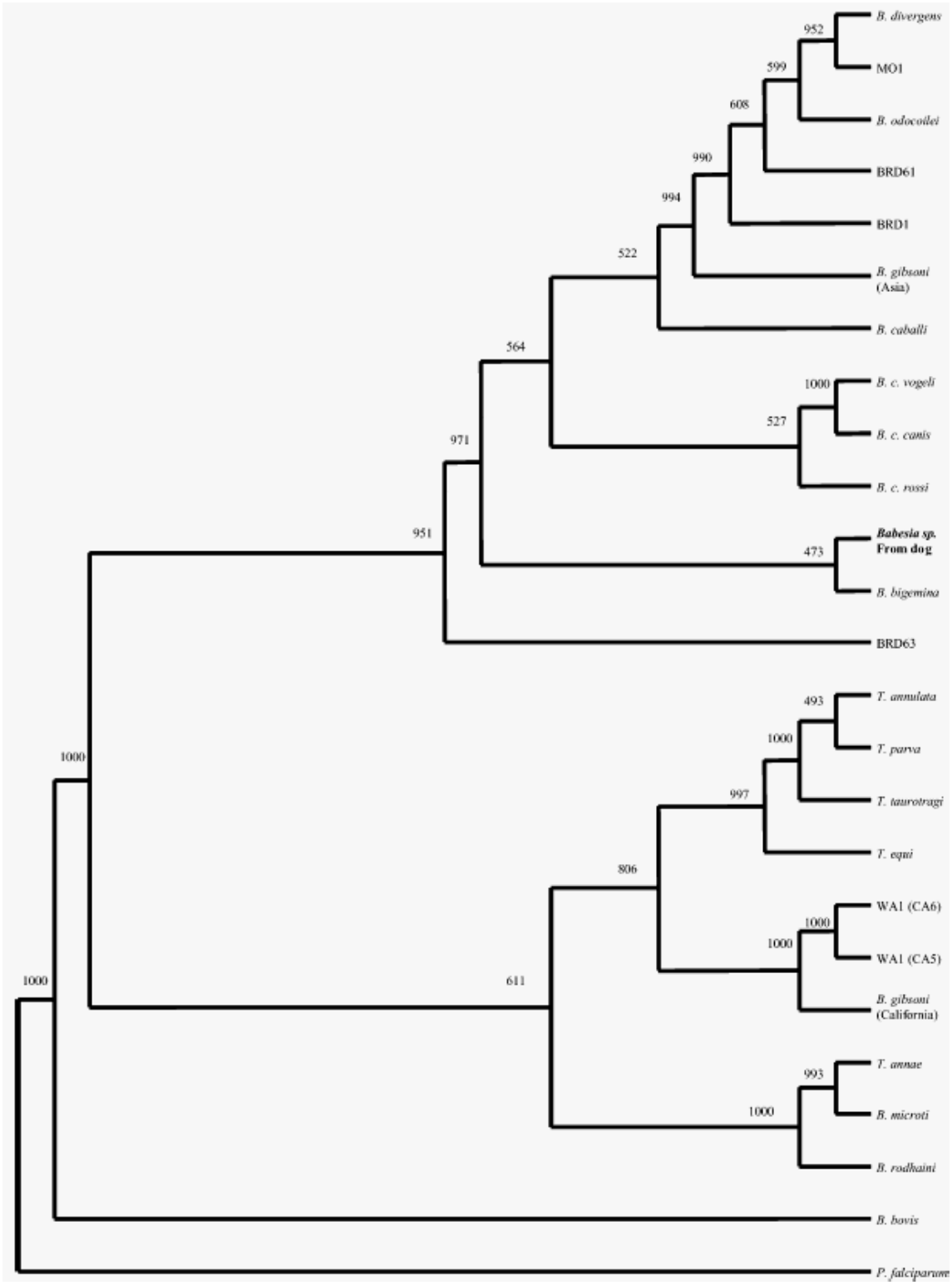

Figure 8. Phylogenetic tree inferred by distance methods using edited alignment of 18S rDNA sequences. The number on each branch shows the occurrence in 1000 bootstrap replicates (Birkenheuer et al, 2004).

B. gibsoni occurs in Asia, North America, northern and eastern Africa, Australia and Europe (Birkenheuer et al., 1999; Muhlnickel et al., 2002; Criado-Fornelio et al., 2003a). Recent genetic characterisations demonstrated that small canine piroplasms also represent a greater diversity than previously thought (Zahler et al., 2000b,c; Kjemtrup et al., 2000; Kocan et al., 2001). One of the recently identified small piroplasms, *Theileria annae* (Zahler et al. 2000b) is phylogenetically closer to *B. microti* than to *B. gibsoni* (Fig. 8) and it can be found with a high frequency among Spanish dogs (Camacho et al., 2001). Criado-Fornelio et al. (2003b) described another small piroplasm, namely *Theileria equi*, from the blood of dogs in Spain using polymerase chain reaction (PCR) and sequencing.

The presence of small babesiae of dogs in Europe was in doubt until the end of the 1980s. Although some cases have been described recently, knowledge of the veterinary and zoonotic importance of these parasites is still very limited (Casapulla et al., 1988, Zahler et al., 2000b,c; Suarez et al., 2001, Camacho et al., 2001, 2003; Criado-Fornelio et al., 2003a,b).

Canine babesiosis was first described in Hungary by Wetzl (1905) who used the name *Piroplasma canis* for the pathogen found in the blood smears of two hunting dogs which visited the county Tolna. The disease was diagnosed again in three dogs from Tolna in the early 1930s (Miklósi, 1931,1932) and in six dogs in the 1970s (Horváth and Papp, 1974) originating from the south-west bank of lake Balaton. Janisch (1986) proved with experimental infections that *D. reticulatus* (syn. *D. pictus*) is the biological vector of *B. canis* in Hungary. He reported that 70 registered cases had been known until 1986. Since then, it has been a severe and frequent disease in the country (Horváth and Papp, 1996). Csikós et al. (2001) diagnosed babesiosis in 1384 dogs between 1992 and 1999 in Szekszárd and its vicinity. Identification of the pathogen has always been based merely on size and morphology of the intraerythrocytic parasites, and no evidence has been found concerning the subspecies of the large canine *Babesia* in Hungary. To the best of our knowledge, confirmed cases of *B. gibsoni* or other small babesiae in dogs have not been reported in Hungary so far.

The detection of both small and large genetically unique canine babesiae that are clinically and morphologically indistinguishable from known piroplasms highlights the need for molecular diagnostics in clinical medicine (Cacciò et al., 2002; Kjemtrup et al., 2000; Zahler et al., 2000b,c). To date, eight genetically different piroplasms have been described in symptomatic dogs (Fig. 7) which resulted in a major change in the concepts of epidemiology and diagnosis of canine babesiosis. Recent advances in molecular methodology (e.g. automated DNA sequencing) have made it possible to detect and identify babesiae with greater sensitivity and specificity than

traditional methods allowed (Birkenheuer et al., 2003; Jefferies et al., 2003). Beside PCR-RFLP (PCR and Restriction Fragment Length Polymorphism) (Zahler et al., 1998; Carret et al., 1999) and reverse line blot hybridization (Matjila et al., 2004), sequencing single PCR products remains a reliable and quick diagnostic method (Criado-Fornelio et al., 2003b). For these reasons, considerable changes in the nomenclature, taxonomy and phylogeny of canine piroplasms can be anticipated in the near future.

2.2.2. Spirochetes

Spirochetes are well studied prokaryotes because of their importance in Lyme disease, the most frequent tick-borne human infection in the northern hemisphere (Brouqui et al., 2004). The disease-causing bacteria are flexible, spiral-shaped, Gram-negative spirochetes with internal flagella and belong to *Borrelia burgdorferi* sensu lato complex (Spirochaetaceae). They have been divided into at least 12 genospecies (Michel et al., 2003; Richter et al., 2004). Six of them: *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii, B. bissettii, B. lusitaniae* and *B. spielmani,* have an unambiguous pathogenic role in human Lyme disease in Central Europe (Strle et al., 1997; Postic et al., 1998; Collares-Pereira et al., 2004; Richter et al., 2004). Several *Ixodes* species can transmit these spirochetes to a large number of avian and mammal hosts (Hillyard, 1996). A considerable number of studies examined the occurrence of Lyme disease spirochetes in ticks in Europe. Hubalek and Halouzka (1998) reviewed the literature of European surveys on *B. burgdorferi* s.l. infection of the most important vector, *I. ricinus*. They showed that the spirochete was present in all *I. ricinus* populations in Europe wherever it was examined. There have been surveys in Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, Finland, France, Germany, Great Britain, Ireland, Italy, Lithuania, The Netherlands, Norway, Poland, Russia, Serbia, Slovakia, Slovenia, Spain, Sweden and Switzerland (Hubalek and Halouzka, 1998). Lakos et al. (1991) investigated the presence of the Lyme disease spirochetes in 31 field collected adult *I. ricinus* ticks in Hungary. Five of them contained *Borrelia burgdorferi* s.l. Spirochetes were successfully cultivated in four cases, detected by immunofluorescence and dark field microscopy as well in two ticks. Two of the isolated strains were tested by Western blot. These studies were not continued later.

From the epidemiological point of view dogs have been very important since they had been declared an effective factor of spreading human borreliosis (Eng et al., 1988). *Borrelia* seropositivity is common among dogs because many of them carry a persistent infection for life and only a fraction of infected animals (~5%) enter the disease status (Levy and Magnarelli, 1992; Beugnet, 2002). Persistent infection even after antibiotic therapy is reportedly common in dogs, because the spirochetes are sequestered in the skin, connective tissue, joint and central nervous

system. Thus, reactivation of infection with recrudescence of disease can occur e.g. in immunocompromised individuals or a co-infection (Shaw et al., 2001). Clinical manifestations of the disease have also been reported in dogs (Greene, 1991). A variety of clinical symptoms (fever, lethargy, lymphadenopathy, kidney disorders, heart block and neuroborreliosis) can be observed (Lissman et al., 1984; Kornblatt et al., 1985). Recurrent polyarthritis is the most frequent manifestation in seropositive animals (Greene, 1991).

Pathogenicity has been firstly proved for *B. burgdorferi* s.l. in North America (Appel et al., 1993). *B. burgdorferi* s.s. and *B. garinii* are found commonly in naturally exposed dogs in Europe and mixed infection with four genospecies (*B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, and *B. valaisiana*) may also occur (Hovius et al., 1998). Speck et al. (2001) suggested that there are some differences in clinical symptoms between dogs having borreliosis in Europe and dogs experimentally infected with *B. burgdorferi* s.s. in the USA.

Canine borreliosis was first described in the USA (Lissman et al., 1984; Kornblatt et al., 1985) and recently in almost all western European countries. *B. burgdorferi* s.l. infection of dogs have been recorded in France (Doby et al., 1988), Great Britain (May et al., 1991), Belgium (McKenna et al., 1995), Spain (Delgado and Carmenes, 1995), Slovakia (Stefancikova et al., 1996), Germany (Bauerfeind et al., 1998), the Netherlands (Hovius et al., 1999b), Sweden (Egenvall et al., 2000), and Switzerland (Speck et al., 2001). The latter turned out to be a *B. afzelii* infection in a naturally exposed dog (Speck et al., 2001). Skotorczak and Wodecka (2005) examined blood samples of tick-infected dogs from north-western Poland and revealed the presence of *B. burgdorferi* s.s. DNA. We have no literature data concerning canine borreliosis in Hungary, however, veterinary clinics frequently diagnose canine borreliosis based on clinical signs or serological methods (personal communication).

3. AIMS

In Europe, the number of reports on canine tick-borne diseases has increased in the past few years (Shaw et al., 2001; Camacho et al., 2001; Chandoga et al., 2002; Criado-Fornelio et al., 2003a,b; Kenny et al., 2004a). Increased national and international mobility of pets (Glaser and Gothe, 1998), the ability of ticks to find niches in new climatic conditions, growing accessibility of natural environments and an increase in the population of wild host species that now have a closer association with human activity (Csányi, 2005) are reported to be the main reasons (Shaw et al., 2001). In Hungary, we have had very limited information concerning tick infestation and tick-borne pathogens of dogs. For these reasons, we started to study the tick species and tick-borne pathogens infecting dogs in our country. Our main aims were the following:

- To design a practical identification key for the adult tick species occurring on dogs in Europe.
- To examine the tick fauna of dogs and the geographical and seasonal distribution of tick species infesting dogs in Hungary with special emphasis on *D. reticulatus*.
- To collect data on tick species living in the environments where dogs can be infested with special emphasis on places where canine babesiosis occurs.
- To ascertain whether small babesiae can occur in dogs in Hungary.
- To carry out a molecular survey on *B. canis* infection of dogs in Hungary in order to clarify the subspecies and obtain detailed information on the geographical and seasonal occurrence of this piroplasm. We also aimed at detecting and identifying *Babesia* sp. from *D. reticulatus* specimens fed on dogs and collected from field.
- To detect and identify *Borrelia* sp. occurring in the blood of dogs and in *I. ricinus* specimens fed on dogs and collected from field.

4. STUDIES

4.1. Hard tick infestation of dogs

4.1.1. Materials and methods

4.1.1.1. Identification key of tick species infesting dogs in Europe

Species for the identification key were selected on the basis of literature search. The two selecting criteria of tick species for our study were: (1) occurrence in Europe and (2) recorded infestation of dogs. A primary search was carried out in the Nuttall Tick Collection (Department of Entomology, Natural History Museum, London UK) which contains several thousands of specimens that was listed by Keirans (1984) according to host species. This is one of the geographically widest and best quality collections accessible on hard ticks. Faunistic surveys (Liebisch, 1984; Grandes, 1986; Beichel et al., 1996; Papadopoulos et al., 1996; Ogden et al., 2000; Farkas and Földvári, 2001; Papazahariadou et al., 2003; Földvári and Farkas, 2005a), monographs and reviews (Hillyard, 1996; Camicas et al., 1998; Estrada-Peňa et al., 2004) have also been examined in respect to the two criteria. Cases where tick infestations were only accidental (e.g. single observation in one report) were not included.

Hard tick specimens used for drawing and examining were unengorged adults kept in 70% ethanol (except for *I. canisuga* and *H. parva* for which only partly engorged females were available) from the tick collection of the Natural History Museum, London, UK. Type specimens were used for identifying generic and specific characters. All morphological features were examined which had been used previously in the description of the earlier publications (Nuttall and Warburton, 1911 and 1915; Arthur, 1960; Babos, 1964; Hillyard, 1996; Walker et al., 2000; Estrada-Peňa et al., 2004) but only those characters which are relatively easy to distinguish were included into our key. A schematic drawing was prepared by Paul D. Hillyard and Gábor Majoros to show the most relevant morphological signs from dorsal and ventral view of both males and females. Drawings have been also made to each genera and species enabling the recognition of the most important morphological features. Dichotomy was avoided wherever it made the identification easier and only essential characters were mentioned in the keys.

4.1.1.2. Collection of ticks from dogs

After previous consultations, 40 veterinary clinics recruited from a wide geographical area within the country were asked to collect hard ticks from dogs which attended the surgeries in a period of four years. A simple questionnaire was designed to investigate the breed and age of host animal, date of collection and habitat to which the dog had been exposed. Questions on previous acaricidal and/or antibabesial treatment(s) and on symptoms of babesiosis were also asked. Ticks were removed from the dogs during the clinical examination and the questionnaires were completed. Specimens collected from each dog were stored in separate labelled tubes containing 70% ethanol. The samples with the questionnaires were posted to the Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent István University, Budapest.

Counting and identification of species, life stage and sex of ticks were carried out under a stereomicroscope. Standard keys of Babos (1965), Hillyard (1996), Estrada-Peňa et al. (2004) were used for species identification of adults; immature specimens were not identified to species level.

4.1.1.3. Collection of ticks from field

Ticks were collected from the vegetation of 32 different locations including ten (Győr-Moson-Sopron, Vas, Veszprém, Zala, Somogy, Baranya, Pest, Csongrád, Borsod-Abaúj-Zemplén and Heves) counties and six districts of Budapest. Most locations were chosen because frequent infestation of dogs and/or occurrence of canine babesiosis were reported (personal communication). A handle was fixed to a white flannel towel (Fig. 9) in order to use the dragging method for collecting ticks (Hillyard, 1996). Larger towels were used for the undergrowth and smaller, flag-like towels for bushes and shrubs. The coloration of the towel made it easier to find the crawling ticks on it. Field collections were done in April-July and September-November during the main activity peaks of ixodid ticks in Hungary. In order to increase the chance of finding tick specimens, collections were usually carried out in the morning (7-10 a.m.) or in the afternoon (3-7 p.m.) when the relative humidity is usually higher. Dragging lasted for approximately one hour at every field collection sites. Temperature and relative humidity on the ground level was recorded with a Datalogger (Gemini, UK). Characteristic flora and water supply of the area were registered as well. Time and location of collection were also recorded when tick specimens were found on vegetation or on the clothes of the collector.

Storing and identification of specimens were the same described for ticks originating from dogs.

Figure 9. Collection of ticks from the vegetation with dragging method.

4.1.2. Results

4.1.2.1. Identification key of tick species infesting dogs in Europe

Sixteen hard tick species of five genera were found in literature, which are known to occur regularly on dogs in Europe (Table 1). It is important to note that *Dermacentor niveus* was excluded. This is regarded as a valid species name by some authors (Horak et al., 2002) and as a synonym of *D. marginatus* by others (Estrada-Peňa, 1990; Hillyard, personal communication). Because these two species do not have major morphological difference and occupy the same geographical range (Arthur, 1960), we did not make a differentiation of them. A key was designed to distinguish between stages, sexes and genera. Adults of the 16 species were included in an illustrated identification key below. The adults' most relevant morphological structures used for identification can be seen in Fig.10.

Table 1. Hard tick species infesting dogs in Europe (Keirans, 1984; Liebisch, 1984; Grandes, 1986; Beichel et al., 1996; Hillyard, 1996; Papadopoulos et al., 1996; Camicas et al., 1998; Ogden et al., 2000; Papazahariadou et al., 2003; Estrada-Peňa et al., 2004).

Valid name	**Synonym**
Ixodes canisuga (Johnston, 1849)	*I. vulpinus* Schulze, 1937
	I. melicola Schulze and Johnston, 1930
	I. sciuricola Schulze, 1933
	I. barbarossae Schulze, 1937
Ixodes ricinus (Linnaeus, 1758)	
Ixodes hexagonus Leach, 1815	*I. autumnalis* Leach, 1815
	I. hexagonus hungaricus Babos, 1964
	I. vulpis Pagenstecher, 1861
Haemaphysalis inermis Birula, 1895	
Haemaphysalis concinna Koch, 1844	
Haemaphysalis punctata Canestrini and Fanzago, 1878	*H. cinnabarina punctata* Canestrini and Fanzago, 1878
Haemaphysalis parva (Neumann, 1897)	*H. otophila* Schulze, 1918
Rhipicephalus bursa Canestrini and Fanzago, 1878	
Rhipicephalus pusillus Gil Collado, 1938	
Rhipicephalus turanicus Pomerantsev, 1936	
Rhipicephalus sanguineus (Latreille, 1806)	
Dermacentor reticulatus (Fabricius, 1794)	*D. pictus* Hermann, 1804
Dermacentor marginatus (Sulzer, 1776)	*I. hungaricus* Karpelles, 1893
	D. niveus Neumann, 1897
Hyalomma aegyptium (Linnaeus, 1758)	
Hyalomma marginatum marginatum Koch, 1844	*H. marginatum* Koch, 1844
Hyalomma marginatum rufipes Koch, 1844	*H. rufipes* Koch, 1844
	H. rufipes rufipes Koch, 1944

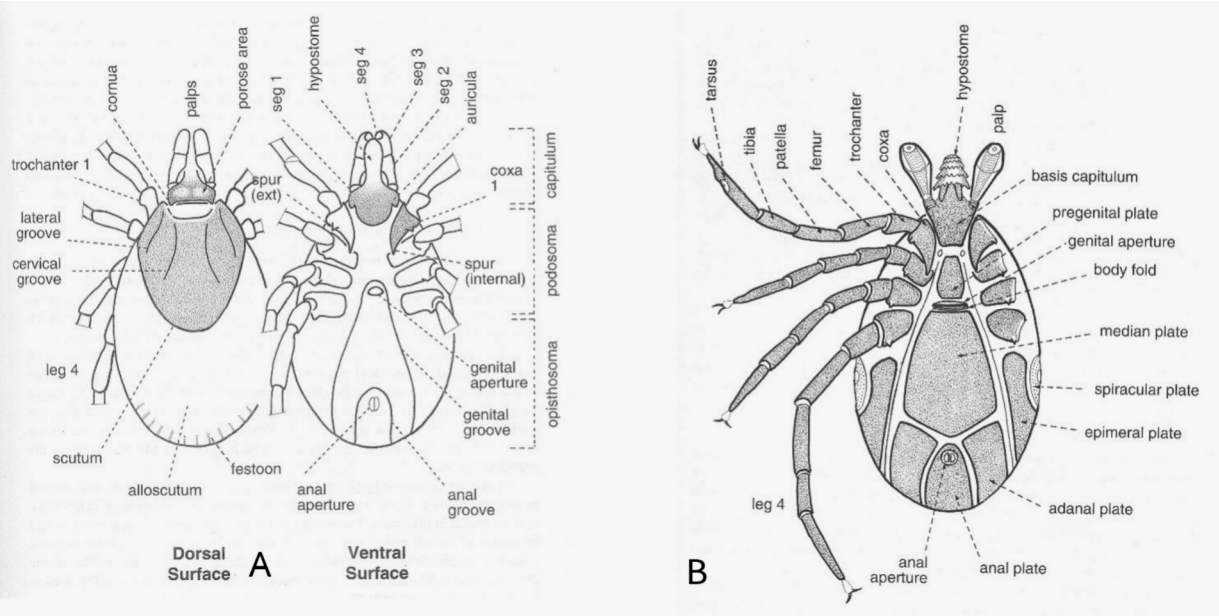

Figure 10. Dorsal and ventral surface of a female (A) and ventral surface of a male (B) hard tick (P.D. Hillyard).

Key to the stages and sexes

1. - Three pairs of legs, spiracles absent.

……..…..**larva**

- Four pairs of legs, spiracles present.

……**2**

2. - Scutum covers entire dorsum of the body.

……**male**

- Scutum confined to anterior of dorsum.

……**3**

3. - Genital opening and porose areas absent.

……**nymph**

- Porose areas and genital opening present.

……**female**

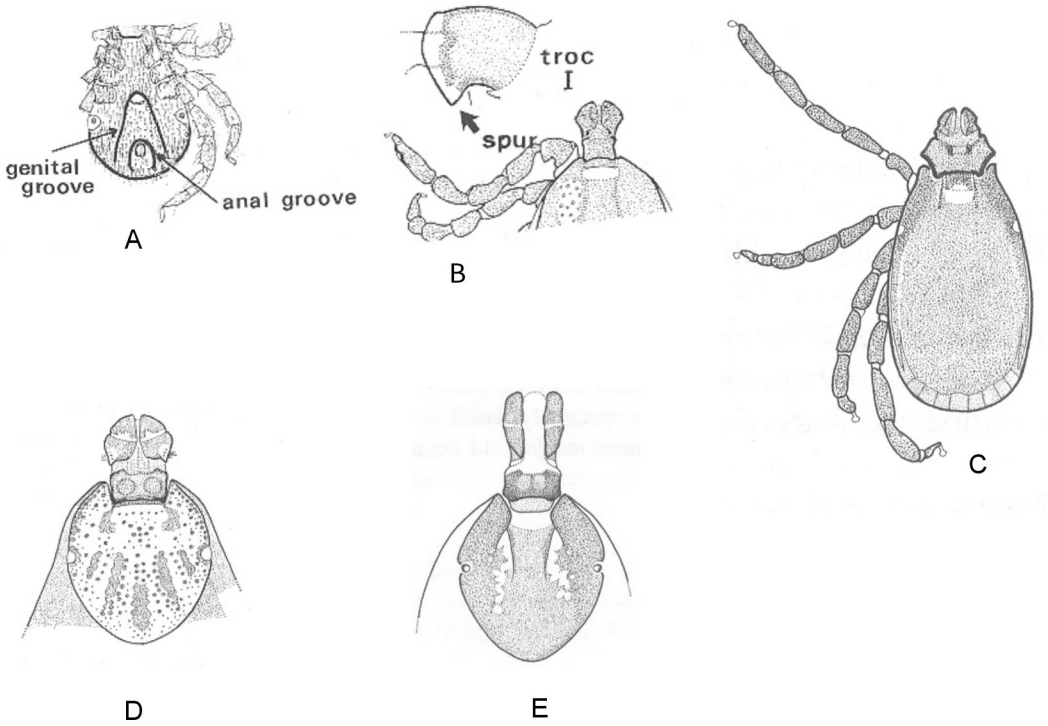

Figure 11. Main characters of *Ixodes* (A), *Haemaphysalis* (B), *Rhipicephalus* (C), *Dermacentor* (D) and *Hyalomma* (E) genera (P.D. Hillyard).

Key to the genera

1. - Anal groove circles anus anteriorly. Scutum without eyes or ornamentation; well-marked festoons absent. (Fig. 11A)

……***Ixodes***

- Anal groove posterior to anus. Scutum with or without eyes and with or without ornamentation; festoons present.

……**2**

2. - Without eyes or ornamentation. Trochanter I with broad spur. External spur on coxa I absent. (Fig. 11B)

……***Haemaphysalis***

- Eyes present. Scutum ornate or not. Trochanter I without broad spur. External spur of coxa I present.

……..……**3**

3. - Scutum inornate. Shape of basis capitulum almost hexagonal in dorsal view. (Fig. 11C)

.....***Rhipicephalus***

- Scutum ornate or not. Shape of basis capitulum subrectangular.

.....**4**

4. - Palps short and wide. Scutum ornate. (Fig. 11D)

.....***Dermacentor***

- Palps longer than wide. Scutum inornate. (Fig. 11E)

.....***Hyalomma***

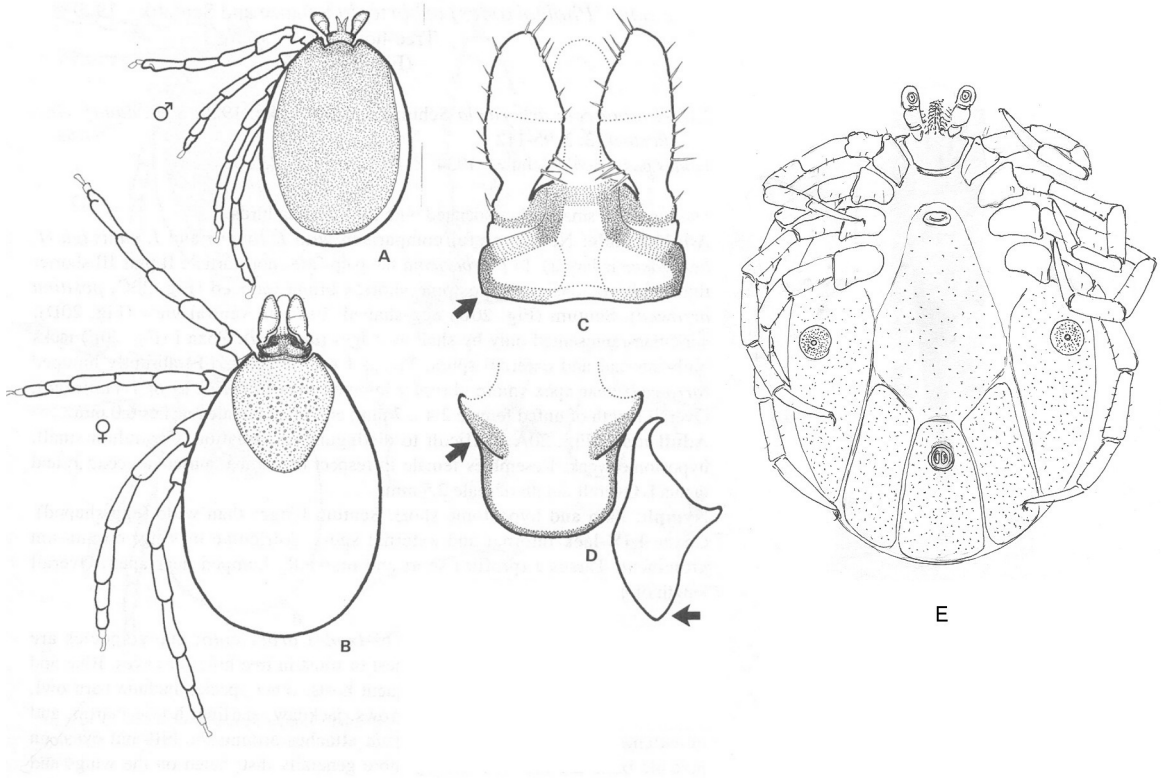

Figure 12. *Ixodes canisuga.* A. Dorsal view of male; B. Dorsal view of female; C. Dorsal view of female capitulum; D. Ventral view of female basis and coxa I; E. Ventral view of male (P.D. Hillyard and G. Majoros).

Key to the species of ***Ixodes*** females

1. - Article II plus III of palp as long as or longer than width of basis capitulum. Scutum broadly rounded posteriorly or hexagonal. Internal spur of coxa I elongate. Location of genital aperture not between coxae II.

.....**2**

- Article II plus III of palp shorter than width of basis capitulum. Scutum narrowly rounded posteriorly. Internal spur on coxa I absent. Location of genital aperture between coxae II.

.....***Ixodes canisuga*** (Fig. 12 B-D)

2. – Scutum broadly rounded posteriorly. External spurs on coxae I-IV short. Location of

genital aperture between coxae IV.

.....***Ixodes ricinus*** (Fig. 13 B-D)

-Scutum hexagonal. External spurs on coxae I-IV absent. Location of genital aperture between coxae III.

…….....***Ixodes hexagonus*** (Fig. 14B-D)

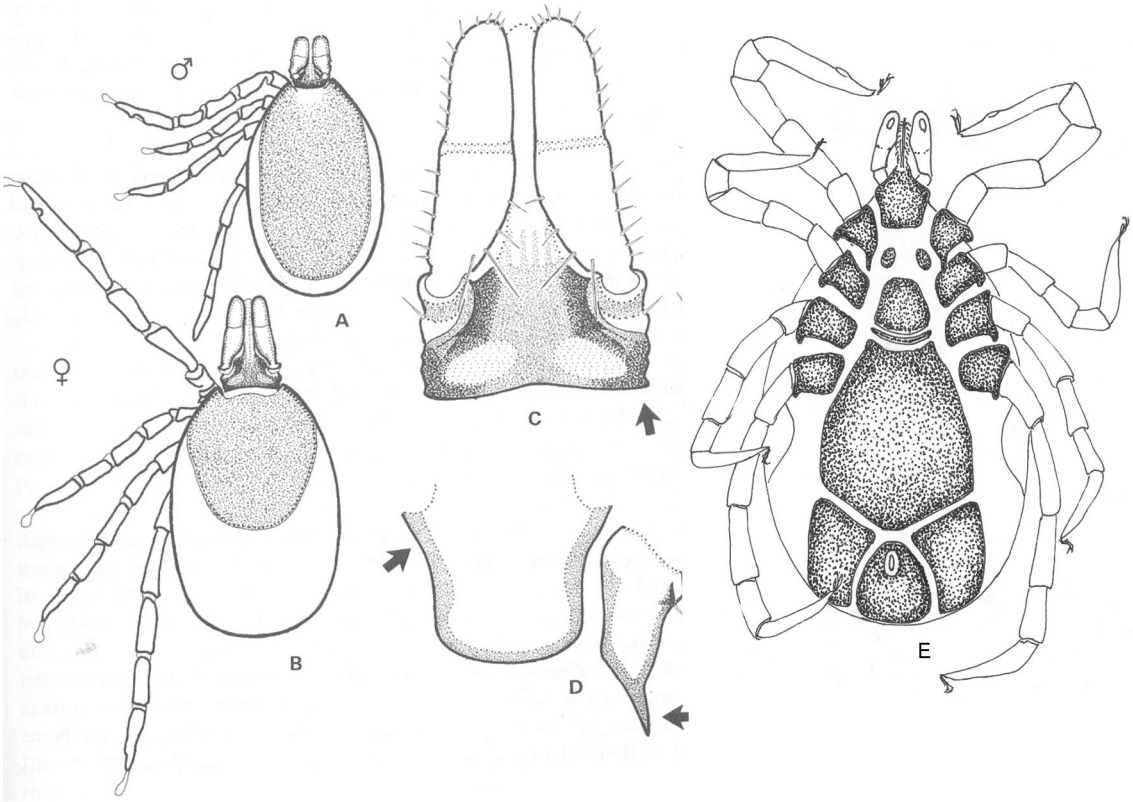

Figure 13. *Ixodes ricinus.* A. Dorsal view of male; B. Dorsal view of female; C. Dorsal view of female capitulum; D. Ventral view of female basis and coxa I; E. Ventral view of male (P.D. Hillyard and G. Majoros).

Key to the species of ***Ixodes*** males

1. - Internal spur of coxa I prominent. Adanal and epimeral plates clearly shorter than median plate. Median plate as long as or longer than wide.

.....**2**

- Internal spur of coxa I short. Adanal and epimeral plates almost as long as median plate. Median plate narrow anteriorly but broad posteriorly.

.....***Ixodes canisuga*** (Fig. 12 A,E)

2. - Internal spurs on coxae II-IV vestigial. Pregenital plate twice as long as broad. Median

plate much longer than wide.

.....***Ixodes ricinus*** (Fig. 13 A,E)

- Internal spurs on coxae II-IV absent. Pregenital plate almost hexagonal. Median plate nearly as long as wide .....***Ixodes hexagonus*** (Fig. 14 A,E)

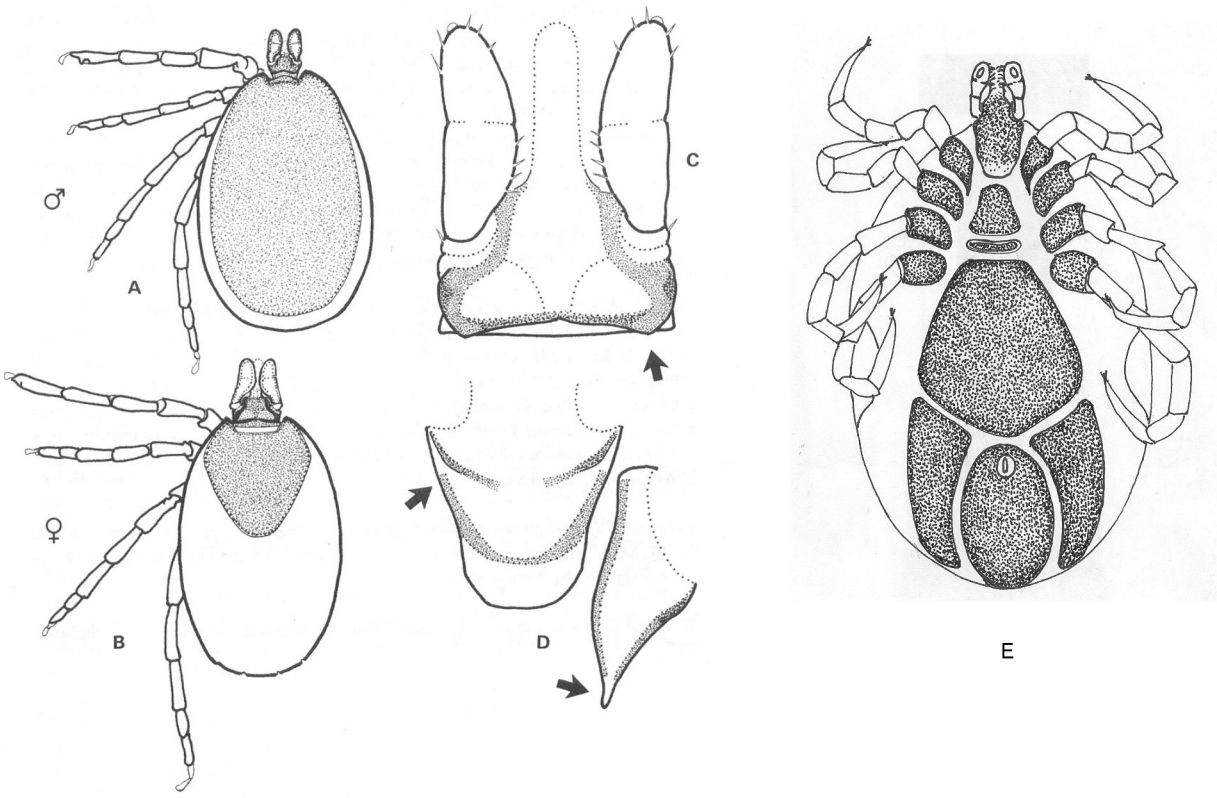

Figure 14. *Ixodes hexagonus.* A. Dorsal view of male; B. Dorsal view of female; C. Dorsal view of female capitulum; D. Ventral view of female basis and coxa I; E. Ventral view of male (P.D. Hillyard and G. Majoros).

Key to the species of ***Haemaphysalis*** females

1. - Article II of palp projects laterally beyond margin of basis capitulum. Spur on trochanter I prominent in dorsal view…… **2**

- Palps do not project laterally beyond basis capitulum. Spur on trochanter I relatively short. .....***Haemaphysalis inermis*** (Fig. 15 B)

2. -Cornua absent. Scutum broader than long. Spur on coxa I short and blunt.

.....**3**

- Cornua present. Scutum almost round. Spur on coxa I prominent.

.....***Haemaphysalis concinna*** (Fig. 16 B)

3. - Spur of coxa IV more prominent than that of coxa I

……***Haemaphysalis punctata*** (Fig. 17 B-E)

- Spur of coxa IV not more prominent than that of coxa I

.....***Haemaphysalis parva*** (Fig. 18 B)

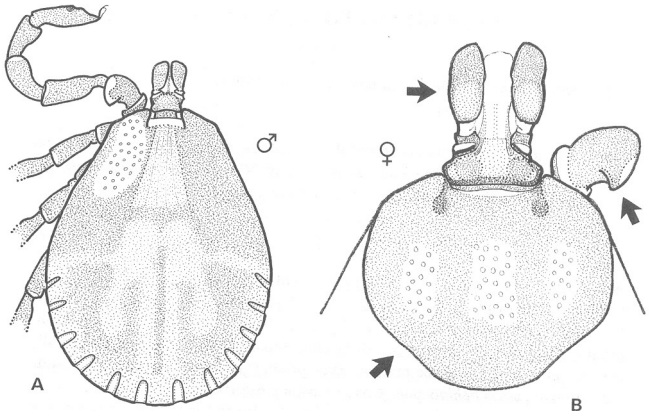

Figure 15. *Haemaphysalis inermis.* A. Dorsal view of male; B. Dorsal view of female capitulum and scutum (P.D. Hillyard).

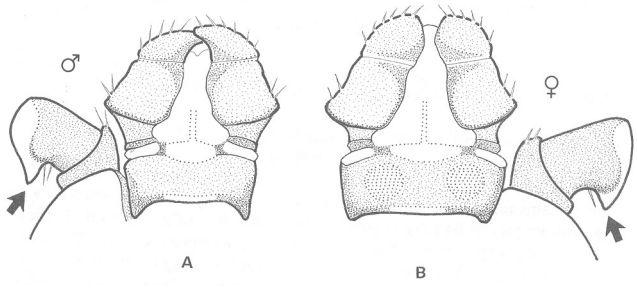

Figure 16. *Haemaphysalis concinna.* A. Dorsal view of male capitulum; B. Dorsal view of female capitulum (P.D. Hillyard).

Key to the species of ***Haemaphysalis*** males

1. - Article II of palp projects laterally beyond margin of basis capitulum. Basis capitulum 1.5x or 2x broader than long. Cornua present. Not all spurs on coxae are small.

.....**2**

- Article II of palp without lateral projection. Basis capitulum narrow. Cornua absent. Spurs on all coxae small.

.....***Haemaphysalis inermis*** (Fig. 15 A)

- 2. Article III of palp curves inward. Basis capitulum at least 2x broader than long. Cornua prominent. Spur on coxa I prominent.

.....***Haemaphysalis concinna*** (Fig. 16 A)

- Article III of palp does not curve inward. Basis capitulum 1.5x broader than long. Cornua blunt. Coxa IV has long, pointed and curved spur.

.....***Haemaphysalis punctata*** (Fig. 17 A,C)

- Article III of palp does not curve inward. Basis capitulum 1.5x broader than long. Cornua blunt. Spurs on coxae short and blunt (lacks a long, pointed and curved spur on coxa IV).

.....***Haemaphysalis parva*** (Fig. 18 A)

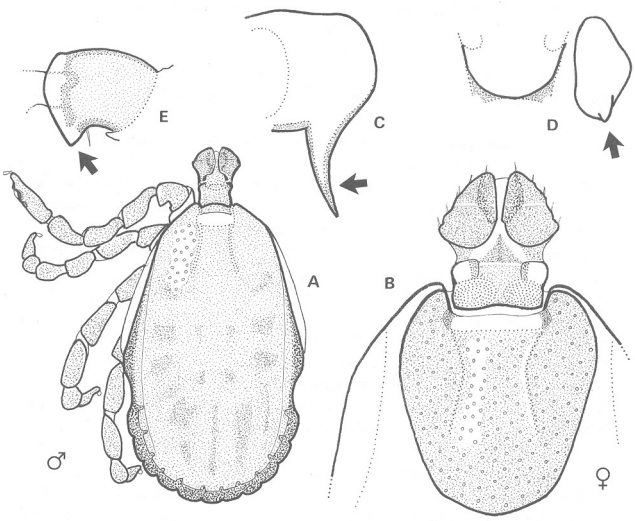

Figure 17. *Haemaphysalis punctata.* A. Dorsal view of male; B. Dorsal view of female capitulum and scutum; C. Ventral view of male coxa IV; D. Ventral view of female coxa I; E. Dorsal view of female trochanter I (P.D. Hillyard).

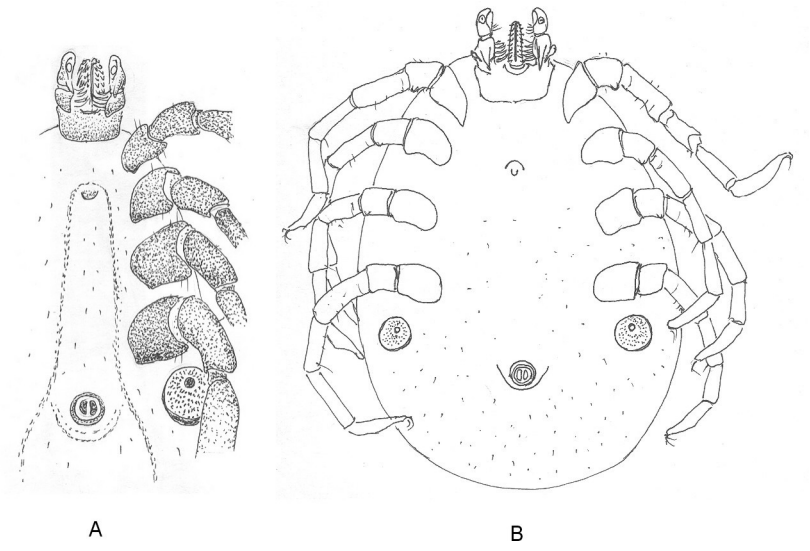

Figure 18. *Haemaphysalis parva.* A. Ventral view of male; B. Ventral view of female (G. Majoros).

Key to the species of ***Rhipicephalus*** females

1. - Large species (3.5 - 4.0mm). Porose areas large and oval, separated by 1x their height. Punctation of scutum fine with sparse larger punctation. Genital aperture V-shaped .....…….....***Rhipicephalus bursa*** (Fig. 19 D,E)

- Small species (2.2 - 2.4mm). Porose areas small, separated by 2x or more their diameter. Punctation of scutum fine with sparse larger punctation. Genital aperture U- shaped ….……..***Rhipicephalus pusillus*** (Fig. 20 C-E)

- Medium-sized species (3.0 - 3.8mm). Porose areas small, separated by 1.5 - 2x their diameter. Punctation of scutum fine with sparse larger punctation. Genital aperture U-shaped…….....***Rhipicephalus sanguineus*** (Fig. 21 B,C)

- Medium-sized species. Porose areas small, separated by 2x their diameter. Punctation of scutum variable but usually dense and conspicuous. Genital aperture U-shaped ……...***Rhipicephalus turanicus*** (Fig. 22 C-E)

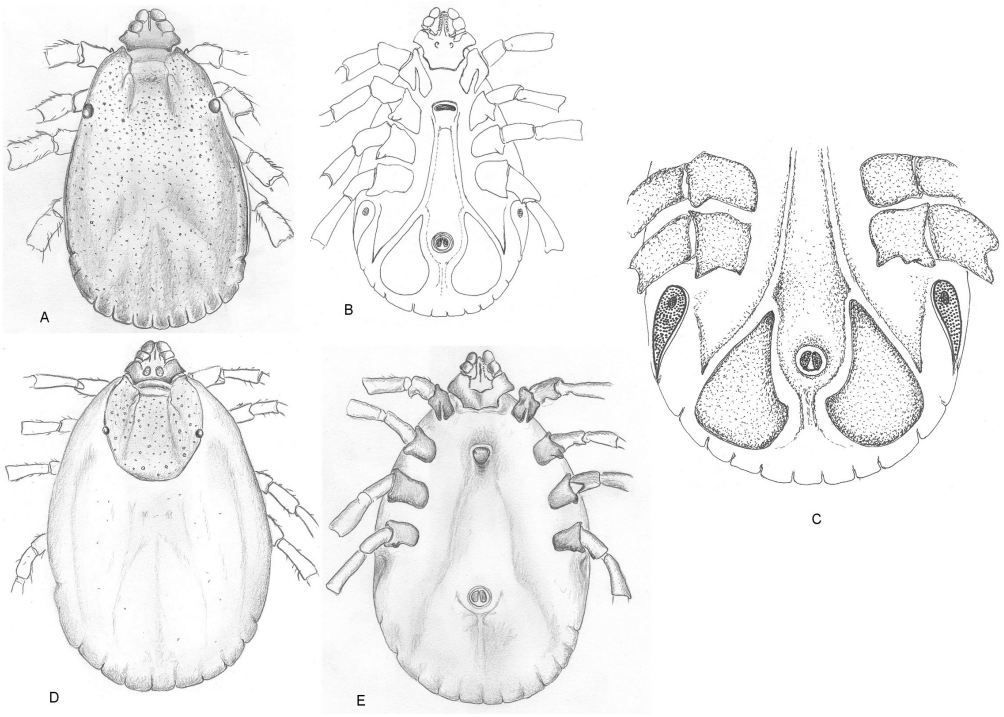

Figure 19. *Rhipicephalus bursa.* A. Dorsal view of male; B. Ventral view of male; C. Ventral view of male adanal plates; D. Dorsal view of female; E. Ventral view of female (G. Majoros).

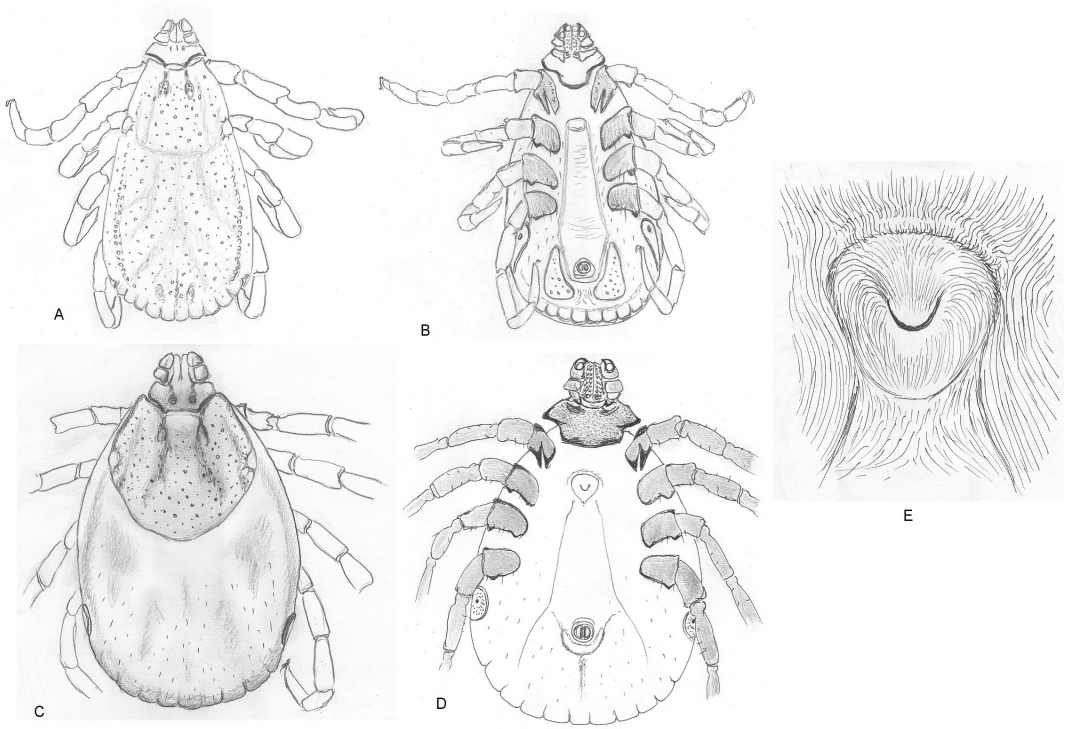

Figure 20. *Rhipicephalus pusillus.* A. Dorsal view of male; B. Ventral view of male; C. Dorsal view of female; D. Ventral view of female of female; E. Ventral view of female of female genital opening (G. Majoros).

Key to the species of ***Rhipicephalus*** males

1. - Large species (3.0 - 4.0mm). Punctation of scutum numerous and fine; a few larger punctations in scapular areas. Adanal plates large, subtriangular with broad posterior. Spiracle broad with long, narrow handle-like extension.....***Rhipicephalus bursa*** (Fig. 19 A-C)

- Small species (1.8 - 2.2mm). Punctation of scutum fine with larger punctations scattered throughout. Adanal plates narrow, curved inwards posteriorly. Spiracle with short, distinctly curved extension…….....***Rhipicephalus pusillus*** (Fig. 20 A,B)

- Medium-sized species (2.7 - 3.3mm). Punctation of scutum usually heavy. Adanal plates vary. Spiracle at least 2x as long as wide…….....***Rhipicephalus turanicus*** (Fig. 22 A,B)

- Medium-sized species (2.5 - 3.2mm). Punctation of scutum ranges from fine to large; usually four more or less regular rows of large punctations visible. Adanal plates elongate triangular with broad posterior. Spiracle shaped like the sole of a slipper……..
.....…….....***Rhipicephalus sanguineus*** (Fig. 21 A)

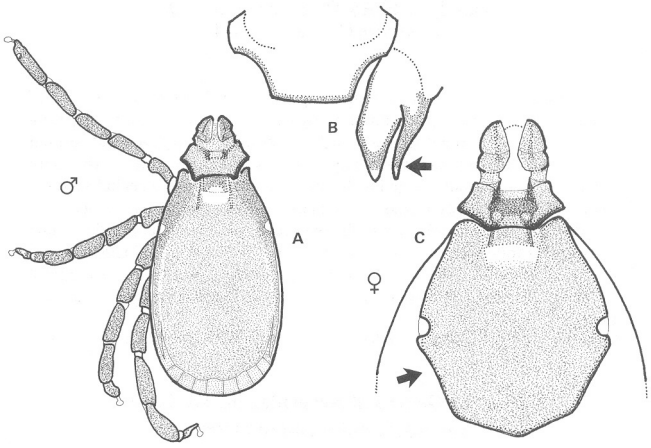

Figure 21. *Rhipicephalus sanguineus.* A. Dorsal view of male; B. Ventral view of female coxa I; C. Ventral view of female capitulum and scutum (P.D. Hillyard).

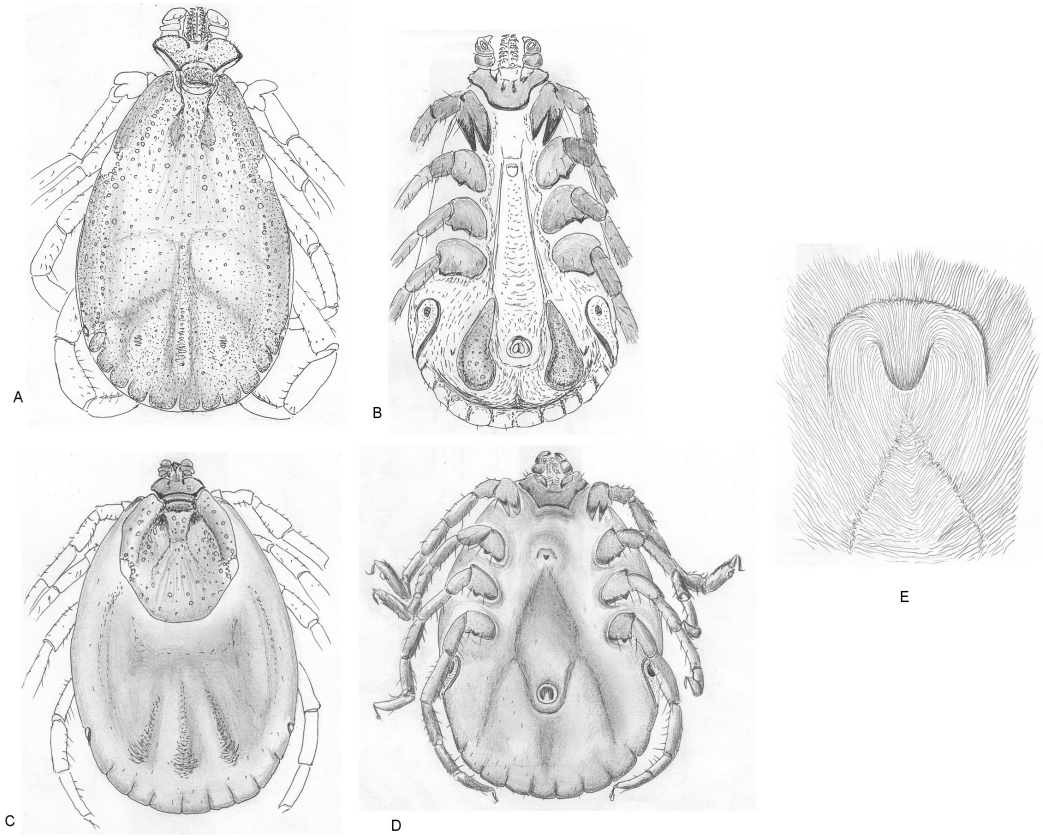

Figure 22. *Rhipicephalus turanicus.* A. Dorsal view of male; B. Ventral view of male; C. Dorsal view of female; D Ventral view of female; E. Ventral view of female genital opening (G. Majoros).

Key to the species of ***Dermacentor*** females and males

1. - Article II of palp with prominent, rear-facing spur. Spur of coxa I not divergent. ..... ***Dermacentor reticulatus*** (Fig 23)

- Article II of palp without spur. Spur of coxa I clearly divergent. ..... ***Dermacentor marginatus*** (Fig. 24)

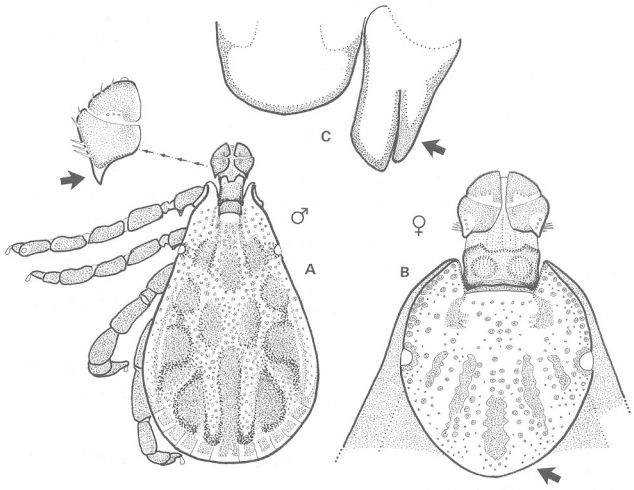

Figure 23. *Dermacentor reticulatus.* A. Dorsal view of male and left palp; B. Ventral view of female capitulum and scutum; C. Ventral view of female basis and coxa I (P.D. Hillyard).

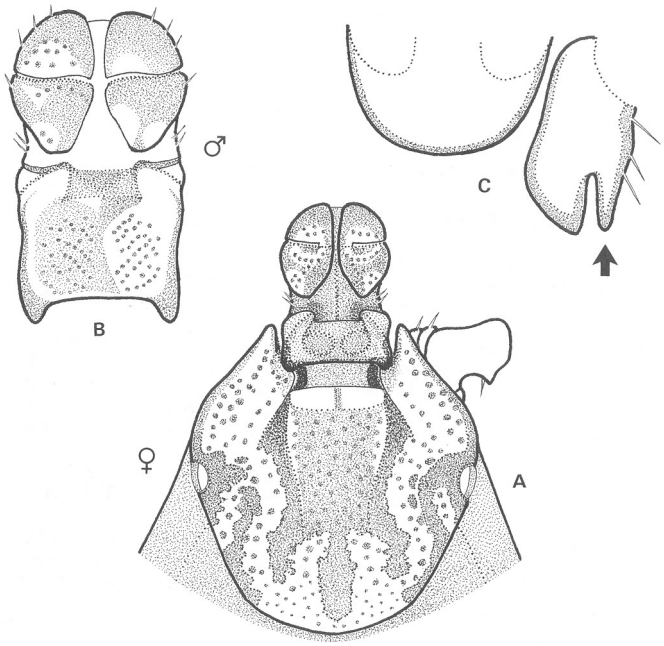

Figure 24. *Dermacentor marginatus.* A. Dorsal view of female capitulum and scutum; B. Dorsal view of male capitulum; C. Ventral view of female basis and coxa I (P.D. Hillyard).

Key to the species of ***Hyalomma*** females

1. - Scutum virtually round in outline; only a few, scattered, large punctations on scutum. Spur on coxa I wide and widely divergent. Genital aperture broadly oval in outline.
.....***Hyalomma aegyptium*** (Fig. 25 B,C)

- Scutum not quite round - outline narrows behind eyes; punctation moderate to numerous, variable in size. Spur of coxa I long and narrow and narrowly divergent. Genital aperture broadly triangular in outline.....***Hyalomma marginatum marginatum*** (Fig. 26 C-E)

- Scutum not quite round – outline narrows behind eyes; and entirely covered by large punctations. Spur of coxa I long and narrow and narrowly divergent. Genital aperture broadly oval in outline.....***Hyalomma marginatum rufipes*** (Fig. 27 C-E)

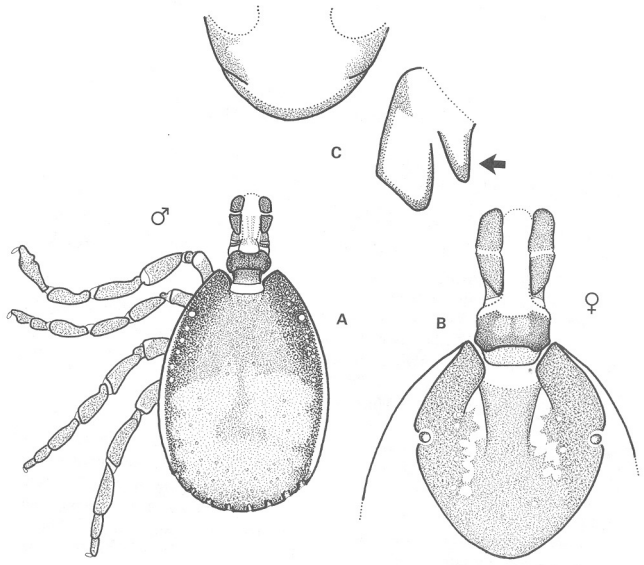

Figure 25. *Hyalomma aegyptium.* A. Dorsal view of male; B. Dorsal view of female capitulum and scutum; C. Ventral view of female basis and coxa I (P.D. Hillyard).

Key to the species of ***Hyalomma*** males

1. - Scutum with only a few, scattered large punctations. Spur of coxa I wide and widely divergent. Adanal plates semi-rectangular, approximately as wide as long and broadest posteriorly. .....***Hyalomma aegyptium*** (Fig. 25 A)

- Surface of scutum smooth with regular, fine punctations and occasional larger punctations. Spur of coxa I long and narrow and slightly divergent. Adanal plates approx. twice as long as wide and separated by a distance equal to their end width.

.....***Hyalomma marginatum marginatum*** (Fig. 26 A,B)

- Surface of scutum rugged with many large punctations. Spur of coxa I long and narrow and slightly divergent. Adanal plates approx. twice as long as wide and separated by a distance clearly less than their end width.

.....***Hyalomma marginatum rufipes*** (Fig. 27 A,B)

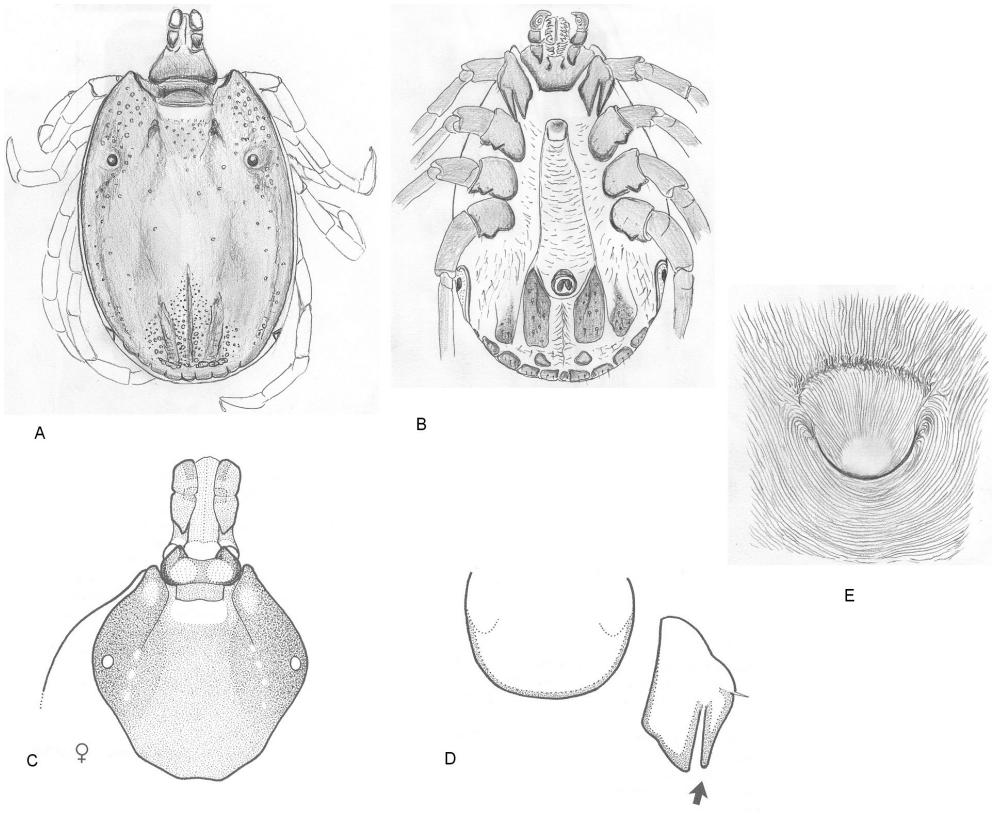

Figure 26. *Hyalomma marginatum marginatum.* A. Dorsal view of male; B. Ventral view of male; C. Dorsal view of female capitulum and scutum; D Ventral view of female basis and coxa I; E. Ventral view of female genital opening (P.D. Hillyard and G. Majoros).

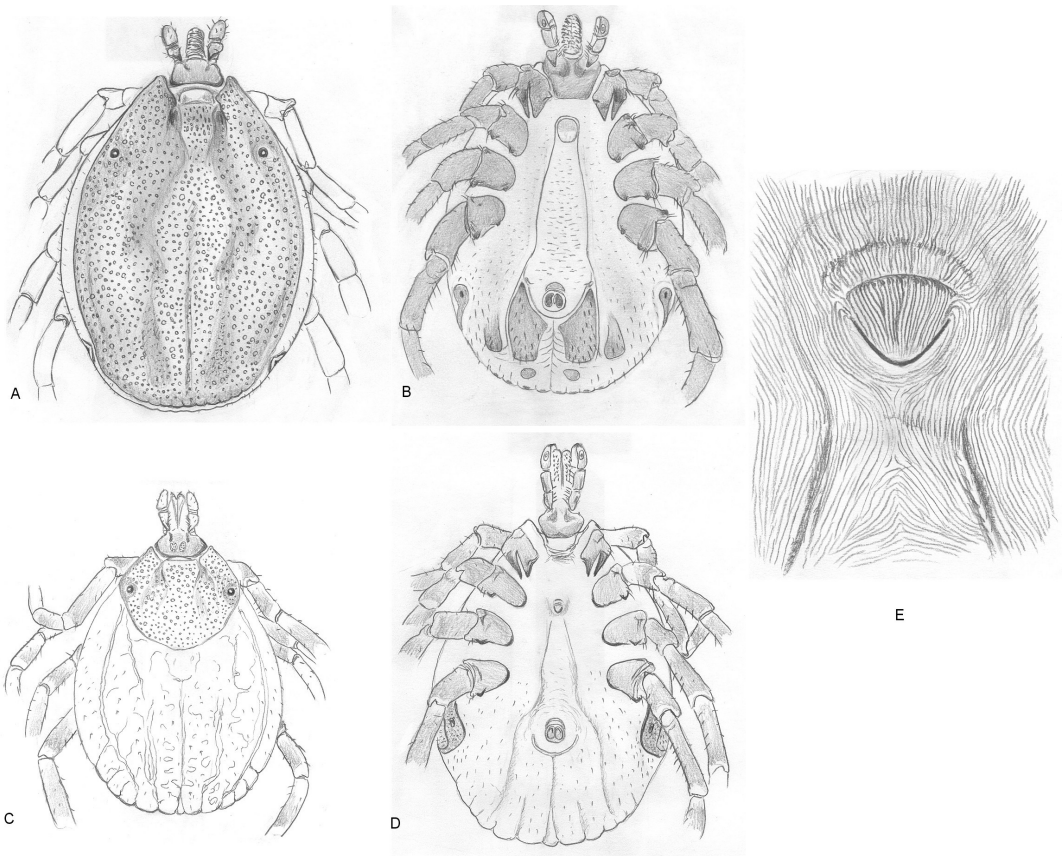

Figure 27. *Hyalomma marginatum rufipes.* A. Dorsal view of male; B. Ventral view of male; C. Dorsal view of female; D Ventral view of female; E. Ventral view of female genital opening (G. Majoros).

4.1.2.2. Collection of ticks from dogs

In 29 veterinary clinics from six districts of Budapest and 13 counties 1779 tick specimens were collected from 606 dogs. Infested animals originated from 55 different locations in the country (Fig. 28). Most hosts were usually infested with a single female (Fig. 29) and very few of them had many (up to 78) ticks (Fig. 30). Most of the tick specimens (1666; 93.6%) were adults belonging to six species, the others (113; 6.4%) were nymphs. *I. ricinus* (872; 52.3%) and *D. reticulatus* (708; 42.5%) were the most frequently identified species. Forty-six (2.8%) and 33 (2.0%) adults were *I. canisuga* and *H. concinna,* respectively. There were four specimens of *I. hexagonus,* two of *Ixodes acuminatus* and only one *Dermacentor marginatus*. Most of the adults (1268, 76.1%) were semi-engorged or fully engorged females. Specimens of *D. marginatus*, *I. hexagonus, I. acuminatus* and *I. canisuga* were only females.

Figure 28. Locations where ticks were collected from dogs.

Figure 29. Blood-sucking female tick.

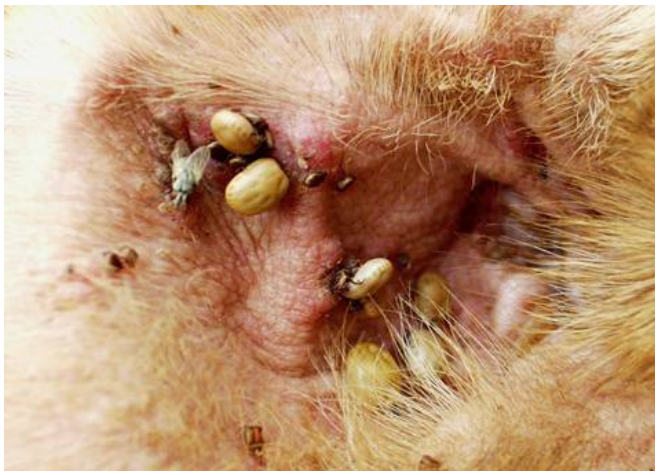

Figure 30. Feeding ticks in a dog's ear.

Single species infestation by either *I. ricinus* or *D. reticulatus* occurred on 281 (46.4%) and 217 (35.8%) dogs, respectively. Mixed infestation caused by these two species was detected on 62 dogs (10.2%). *D. marginatus* and *I. hexagonus* were found in single infestations, while *H. concinna*, *I. canisuga* and nymphs occurred in mixed infestations.

I. ricinus was collected in all locations (Fig. 28). Dogs infested with *D. reticulatus* were found at 42 out of 55 localities (Fig. 31). Based on the date of tick collection records *D. reticulatus* and *I. ricinus* occurred throughout the year (Fig. 32). There was a greater activity peak of these species in March-April and a smaller one in September-October. *I. canisuga* was collected in January and April, *H. concinna* occurred from April to July. *I. hexagonus* was found in April, May and July and *D. marginatus* was removed in June.

Figure 31. Locations where *Dermacentor reticulatus* was found on dogs.

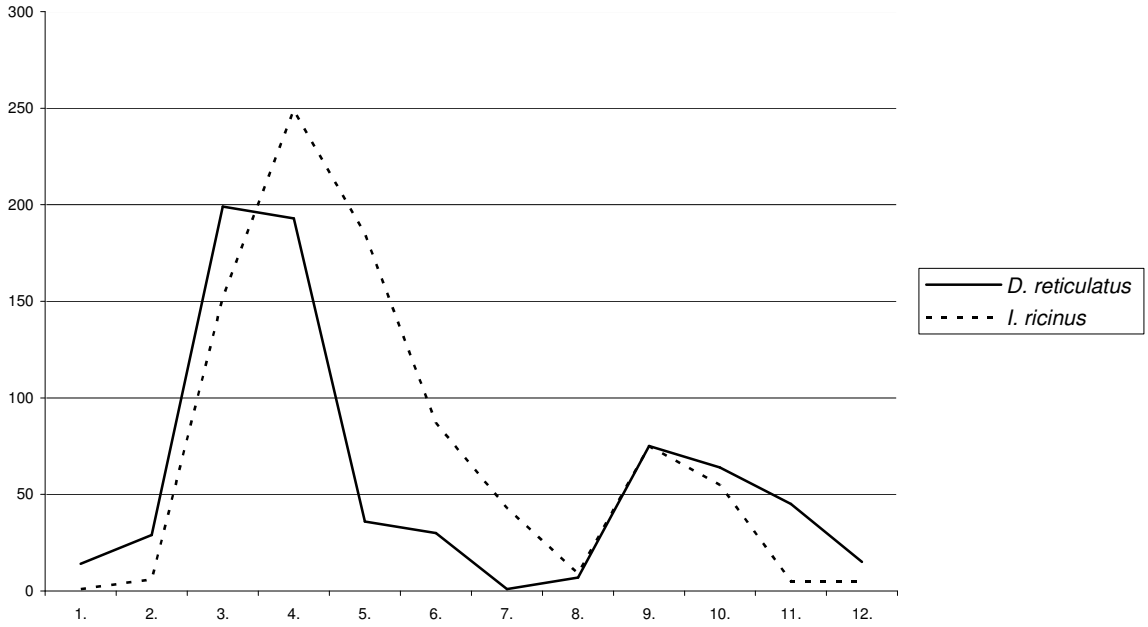

Figure 32. Seasonal occurrence of *D. reticulatus* and/or *I. ricinus* on dogs according to date of collection.

Localization of tick specimens on dogs was recorded in all 180 questionnaires that were returned. The most preferred sites of tick attachment in decreasing order were head, neck and legs. Living conditions of 103 (57.2%) dogs were given in the questionnaires. Two thirds (68; 66%) of the animals lived in gardens, 23 (22.3%) in flats and there were 12 (11.7%) stray dogs. Walking habits for 88 dogs were indicated, which showed that they were walked in watersides (25; 28.4%),

forests (15; 17.1%), mixed habitats (15; 17.1%), meadows (12; 13.6%), parks (5; 5.7%), streets (4; 4.5%), or were living in a garden and not walked at all (12; 13.6%). No association was found between living conditions/strolling and the species of ticks that were collected.

Based on clinical signs (e.g. fever, weakness, lethargy, loss of appetite, haemoglobinuria), canine babesiosis was diagnosed by the veterinarians in 113 (18.6%) tick-infested dogs. Thirty-six (31.9%) of these cases were confirmed by the detection of intraerythrocytic *Babesia* forms in blood smears or amplification of *Babesia* DNA with PCR. Specimens of *D. reticulatus* were collected from 90 (79.6%) dogs having clinical signs of canine babesiosis.

4.1.2.3. Collection of ticks from field

In total 421 tick specimens belonging to five species were collected from field. Ticks were found at 31 out of 32 visited sites (with the exception of Veszprém). Fifteen locations were in Budapest and 16 in other parts of the country (Fig. 33). In eight locations (Németbánya, Csévharaszt, Törökbálint, Baktüttös, Gödöllő, Szokolya, Bükk and Pilis) ticks were accidentally found on the body of the collector and not on the towel. Frequency of occurrence was 1-41 specimen/site. Most (315; 74.8%) of the specimens were adults, the others were nymphs (92; 21.9%) and larvae (14; 3.3%). More than half of the collected ticks were females (181; 57.5%).

Figure 33. Locations where ticks were collected from field (* indicates places where *D. reticulatus* was found and □ where it was not).

Based on the number of collected specimens, *D. reticulatus* was the most frequent species (154; 48.9%). It was found in five out of six districts of Budapest and in eight out of the ten

counties (with the exception of Borsod-Abaúj-Zemplén and Heves) (Fig. 33). This species occurred in habitats with typically well water supply (pond, lake or channel in the vicinity), dense vegetation (reedy areas; meadows and pastures; river banks; railway embankments; outskirts of deciduous forests or cities; rudimentary habitats along roads and paths) (Fig. 34). The temperature was between 16-30 °C and the relative humidity on the ground usually above 50% where *D. reticulatus* was collected.

I. ricinus was the second most common species according to the number of collected specimens (135; 42.9%) and the most commonly occurring being present in all districts of Budapest and all counties where collection was carried out (Fig. 33). It was found in the same habitats as *D. reticulatus* but also on drier, less humid areas. There were 17 (5.4%) specimens of *H. concinna* from counties Pest, Somogy, Zala, Veszprém and Vas. Seven specimens of *D. marginatus* were collected on a sheep run in the vicinity of Nyíregyháza. Two females of *H. inermis* were found in the mountains Pilis and Börzsöny on the cloths of the collector.

Figure 34. Typical living habitat of *D. reticulatus*.

4.1.3. Discussion

4.1.3.1. Identification key of tick species infesting dogs in Europe

Sixteen hard tick species were found during our literature search which commonly occur on dogs in Europe. Since migration of birds and international travelling enable ticks to establish in new habitats, it is possible that this list needs to be updated later. We omitted species which were found only accidentally on dogs or if the canine infestation was not autochthonous in Europe.

Our identification key enables easy and appropriate identification of adult tick species known to occur on dogs in Europe. Its main advantage to other keys is that only a limited number of species have to be taken into consideration during the identification. Furthermore, the comparison and distinction of the 16 species can be achieved with fewer morphological characters then in case of the traditional identification keys (Arthur, 1960, Babos, 1965). The short morphological species descriptions and illustrations focused on the adult stages are intended as identification rather than as comprehensive descriptions. With a few exceptions, determination of tick species can be made most confidently on the basis of unengorged females (Hillyard, 1996). However, attention has to be paid, when an engorged female has to be identified. Some characters on these specimens can be modified (e.g. the position of the genital opening) because of the stretching effect of the blood amount in the intestines. Considering these modifications, the key is useful also for engorged ticks. Male specimens often tend to be poor in specific characters though fortunately males are usually found in the company of females. The nymphs however, can be difficult to identify in the absence of adult specimens even with an identification key (Babos, 1965, Hillyard, 1996). When only larvae are available, more serious difficulties arise because these tiny specimens require a relatively advanced level of microscopy. For these reasons, our key is restricted to the adult stage and we suggest to turn to a specialist or to use molecular biological methods if an immature tick specimen needs to be identified to species level.

Based on morphological (Klompen et al., 1997) or molecular biological data sets (Black et al., 1997; Murrell et al., 1999; Dobson and Barker, 1999; Norris et al., 1999), a series of systematic analyses has provided useful information on the relationships of hard tick subfamilies and species. There are molecular phylogenetic data based on the amplification and analysis of either nuclear (Crampton et al., 1996) or mitochondrial (Black and Piesman, 1994; Caporale et al., 1995; Hubbard et al., 1995; Rich et al., 1995; Mangold et al., 1998) DNA sequences only for some species parasitizing dogs. Mitochondrial 16S rDNA was found to be useful not only to assess phylogenetic relationships of diverse hard and soft ticks (Black and Piesman, 1994) but also to discriminate between species (Caporale et al., 1995). Detailed information on systematics, phylogeny,

biogeography and evolution of hard ticks can be found in reviews from Klompen et al. (2000) and Barker and Murrell (2002). However, there is scant information concerning the molecular analysis of tick species that are usually found on a typical host e.g. dogs. The continuation of our morphological study will be a molecular biological examination of the same species. Since there are already data for 16S mt rDNA sequences in the GenBank® for some of the 16 species, we plan to amplify the missing ones and make a phylogenetic analysis of them.

The simplicity of this key helps in tick identification not only experienced entomologists but also veterinarians who play central role in the diagnosis, treatment and prevention of tick-transmitted diseases of dogs. Several of the tick-borne pathogens can cause serious disease in humans and domesticated animals (Shaw et al., 2001). Because veterinarians play an important role in advising the public as to the zoonotic potential of disease agents transmitted by ticks, they have to be aware of the possible infestation risk exposed by ticks. Therefore, we list the pathogens of humans and dogs which might be carried by these 16 hard tick species (Tables 2-3).

Table 2. Human and/or canine pathogens transmitted by *Ixodes* and *Haemaphysalis* species infesting dogs (Gern et al., 1991; Macaigne and Perez-Eid, 1991; Rehacek et al., 1991; Estrada-Peña et al., 1995; Hillyard, 1996; Juricova et al., 2002; Spitalska and Kocianova, 2003; Sréter-Lancz et al., 2005; N.a.= no literature data available).

Tick species	**Pathogen**
Ixodes canisuga	*B. burgdorferi* s.l. *Pasteurella pestis*
Ixodes ricinus	louping-ill virus tick-borne encephalitis virus *Rickettsia helvetica* *Rickettsia monacensis* *R. conori* *C. burnetii* *Anaplasma phagocytophilum* *B. burgdorferi* s.l. *Francisella tularensis* *Babesia divergens* *B. microti*
Ixodes hexagonus	tick-borne encephalitis virus *R. conori* *B. burgdorferi* s.l. *B. microti*
Haemaphysalis inermis	tick-borne encephalitis virus *C. burnetii*
Haemaphysalis concinna	tick-borne encephalitis virus *C. burnetii* *F. tularensis*
Haemaphysalis punctata	Bhanja virus louping-ill virus Crimean Congo haemorrhagic fever virus tick-borne encephalitis virus *C. burnetii* *F. tularensis* *Listeria monocytogenes* *Brucella abortus*
Haemaphysalis parva	N.a.

Table 3. Human and/or canine pathogens transmitted by *Rhipicephalus*, *Dermacentor* and *Hyalomma* species infesting dogs (Beati et al., 1992; Zahler, 1994; Estrada-Peňa et al., 1995; Hillyard, 1996; Oteo et al., 1996; Beati et al., 1997; Lakos, 1997; Raoult et al., 2002; Sonogo et al., 2003; Coutinho et al., 2005; Földvári and Farkas, 2005b; N.a.= no literature data available)

Tick species	**Pathogen**
Rhipicephalus bursa	*Anaplasma spp.* *Theileria equi*
Rhipicephalus pusillus	*Rickettsia* spp.
Rhipicephalus turanicus	*Rickettsia* spp.
Rhipicephalus sanguineus	*Rickettsia canis* *R. conorii* *Anaplasma platys* *Salmonella* spp. *E. canis* *H. canis* *B. canis canis* *B. canis vogeli* *B. gibsoni* *Leishmania chagasi*
Dermacentor reticulatus	Bhanja virus, Palma virus tick-borne encephalitis virus *R. conori* *R. sibirica* *R. slovaca* *R. rickettsii* *R. prowazekii* *C. burnetii* *F. tularensis* *B. abortus* *L. monocytogenes* *Yersinia pseudotuberculosis* *Babesia divergens* *B. canis canis* *T. equi*
Dermacentor marginatus	Bhanja virus tick-borne encephalitis virus *R. conori* *R. slovaca* *C. burnetii* *B. canis canis*
Hyalomma aegyptium	N.a.
Hyalomma marginatum marginatum	Sindbis virus West Nile virus Bahig virus, Omsk virus Crimean-Congo haemorrhagic fever virus *Rickettsia aeschlimannii* *B. abortus* *B. canis* *T. equi*
Hyalomma marginatum rufipes	Crimean-Congo haemorrhagic fever virus *R. conorii*

4.1.3.2. Collection of ticks from dogs and from field

1779 specimens of seven hard tick species were found on 606 dogs in 29 veterinary clinics of Hungary. More than 90% of them were adults, which can be partly explained by the macroscopic examination of the animals that may have overlooked nymphs and larvae. 421 tick specimens belonging to five species were collected from field at 31 sites. We collected 1 to 41 specimens in each selected location (15 in Budapest and 16 in other parts of the country).

Based on the number of infested dogs, *I. ricinus* was found to be the most prevalent species. This finding is in accordance with German and British studies (Beichel et al., 1996; Ogden et al., 2000). During our field collection *I. ricinus* was the second most common species according to the number of collected specimens (135; 42.9%) and the most commonly occurring being present in all districts of Budapest and all counties where collection was carried out. Compared to other European countries (Hillyard, 1996), the frequent occurrence of *I. ricinus* in the field collections seems to be a general trend. *I. ricinus* had been reported to be common in Hungary already in the middle of the 20th century (Babos, 1965, Janisch, 1959). It is widespread in Europe and has a wide host range (Hillyard, 1996). It has great medical and veterinary importance as a vector of Lyme disease spirochete *Borrelia burgdorferi* s.l. (Beichel et al., 1996), *Ehrlichia* spp. (Cinco et al., 1997), tick-borne encephalitis virus (Jaenson et al., 1994; Beugnet, 2002) and other disease agents (Table 2). Lakos (1985) reported first on the occurrence of human Lyme borreliosis in the country. There is an increasing number of *Borrelia* seropositive human patients (Lakos, 1991) and dogs (personal communication), but a survey of canine borreliosis or ehrlichiosis has not been conducted in Hungary. *Rickettsia helvetica, Rickettsia monacensis* (Sréter-Lancz et al., 2005) and *Anaplasma phagocytophilum* (Sréter et al., 2004) has been recently detected in *I. ricinus* specimens from Hungary. According to the date of tick collection, *I. ricinus* can infest dogs throughout the year. As a consequence, there is a risk of infection with pathogens transmitted by these species in every season.

D. reticulatus occurred on dogs with the second greatest number. This species is known to infest dogs with a high affinity (Hillyard, 1996) and has been proved to be vector of *B. canis* in Hungary (Janisch, 1986) and in other European countries (Regendanz and Reichenov, 1932; Martinod et al., 1985; Zahler and Gothe, 1997; Zahler et al., 2000a). Until the late 1990`s this species was known to be present only in the middle and western parts of Hungary (Horváth and Papp, 1996). Results of the present study have, therefore, improved our knowledge about the geographical distribution of this tick species, because we collected it also in north-eastern (Nyíregyháza, Mátészalka) and south-eastern (Szeged) parts of the country. Compared to data of similar surveys, *D. reticulatus* seems to infest dogs more often in Hungary than in Spain (Grandes,

1986), Greece (Papadopoulos et al. 1996; Papazahariadou et al., 2003) and in the UK (Ogden et al., 2000). The sampling method could also contribute to the high proportion of this species, because it is probable that more *Babesia*-infected animals (possibly carrying the vector tick) were taken to the veterinary clinics than healthy ones. This hypothesis is supported with the relatively high number of dogs (113; 18.6%) having clinical signs of babesiosis among the examined individuals. In accordance with this, dogs with clinical signs of babesiosis had higher *D. reticulatus* infestation (79.6%) than all dogs examined (56.6%). This species was found to infest dogs throughout the year which can explain the observations of canine *Babesia*-infections during the winter months in Hungary (Csikós et al., 2001). It is also known to transmit tick-borne encephalitis virus, *F. tularensis* and *Rickettsia* spp. (Zahler, 1994) (Table 2).

Unexpectedly, the most common species found in the field was *D. reticulatus*, although, formerly, this species was not reported to be common in field collections (Janisch, 1959; Babos, 1965). This can be partly explained by the fact that collections were carried out on places where we had information on frequent infestation of dogs and occurrence of canine babesiosis. But several other factors might play a role. One of them is the presence of humid natural and semi-natural/semi-urban habitats which are appropriate for this species (Zahler, 1994). Beyond the relatively high humidity, *D. reticulatus* needs to find hosts for all three stages to maintain its life cycle. For larvae and nymphs, all of the common rodent species and rabbits are present in a great number in Hungary (Csányi, 2005). The adults of the species have very broad host range. Not only domesticated but most of the free-living mammals serve as a host (Zahler, 1995). Given the fact, that the populations of wild boar (Fig. 35), red deer, roe deer, and fallow deer increased, while hare, stray dog, stray cat and red fox populations remained at high levels during the last decades (Csányi, 2005), it is possible that this tick species of high adaptability (Meyer-König et al., 2001) is spreading. On the other hand, global warming, deforestation, decreasing use of pesticides may also have an influence. Based on our preliminary data from 31 field collections and the survey on dogs, these may explain the high occurrence of canine babesiosis (Csikós et al., 2001; Földvári et al., 2005; Földvári and Farkas, 2005a and 2005b) in Hungary. However, long-term studies with increased number of sampling sites and specimens would be needed to gain a representative picture of the Hungarian tick fauna.

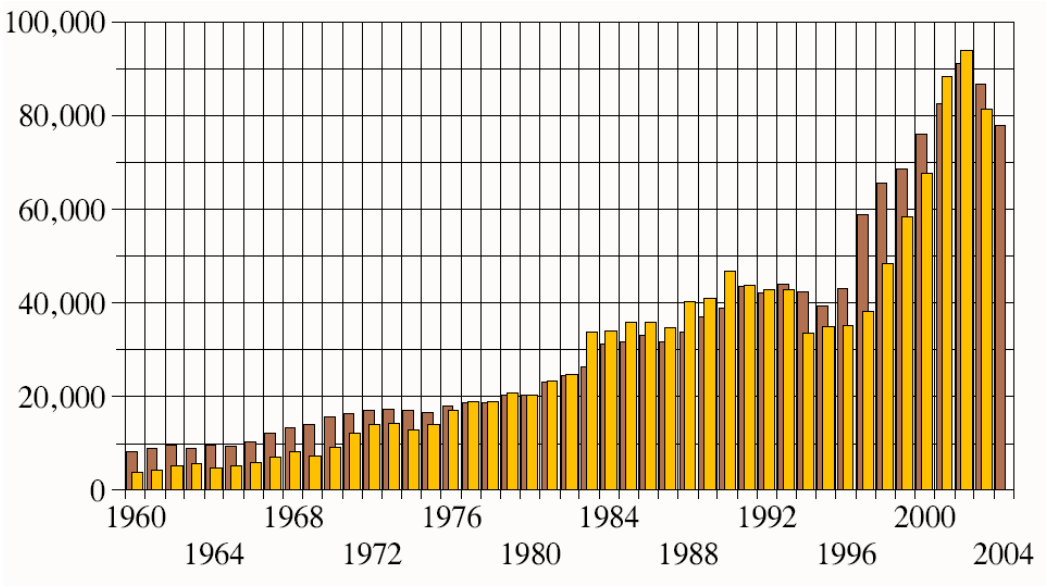

Figure 35. Estimated (brown bars) and harvested (yellow bars) number of wild boar between 1960- 2004 in Hungary (Csányi, 2005).

Forty-six specimens collected from dogs were identified as *I. canisuga.* Babos (1965) reported that this species can also infest dogs in Hungary. It usually parasitizes medium-sized and large mammals, e.g. foxes that regularly return to their nest or lair (Jaenson et al., 1994). *I. canisuga* has been shown to be important vector of *B. burgdorferi* s.l. in areas where *I. ricinus* is absent (Estrada-Pena et al., 1995) (Table 2). We did not find this species with dragging.

H. concinna was also found on dogs that lived in central (Budapest, Nagykovácsi), eastern (Nyíregyháza) and western (Cserszegtomaj, Városlőd) parts of Hungary. Although in small number (n=17), we collected *H. concinna* in five different counties (Somogy, Pest, Vas, Veszprém and Zala) from field. These are partly new geographical data on the occurrence of this species, since Babos (1965) reported that it is likely to occur only in the western half of the country (Transdanube). This species is known to be restricted to areas where the environment is relatively unaltered (Hillyard, 1996). Spitalska and Kocianova (2003) recently showed the ability of this tick species to carry *C. burnetii* (causative agent of Q-fever) in Slovakia and Hungary (Table 2). Boni et al. (1998) reported that dogs can be infected with these intracellular zoonotic bacteria.

H. inermis which is also able to carry *B. burgdorferi* s.l., *C. burnetii* and tick-borne encephalitis (Macaigne and Perez-Eid, 1991; Rehacek et al., 1991; Hillyard, 1996), was found in two field collections (Pilis and Börzsöny mountains). This tick species was reported to be the rarest from this genus in Hungary (Babos 1965). These *Haemaphysalis* species need more attention and further epidemiological studies to be aware of the risk they are posing to human and animal health in our region (Table 2).

I. hexagonus was reported to be a commonly occurring species in Hungary (Babos, 1965) however, based on this study it was found to be less prevalent on dogs compared to Germany (Beichel et al., 1996) and Great Britain (Ogden et al., 2000) and we did not find it during our field collections. *I. hexagonus* usually infest medium-sized and large mammals that have a permanent dwelling, such as carnivores (Jaenson et al., 1994). This species may be involved in the epizootiology of, e.g. *B. burgdorferi* s.l. (Gern et al., 1991) and *T. annae,* a recently identified canine piroplasm in northwest Spain (Camacho et al., 2003) (Table 2).

Two females *of I. acuminatus* infested a dog in the town Veszprém. This species had been previously found once on a hedgehog in Budafok (Babos, 1965). The hosts for all stages of this species are usually small rodents and insectivores (e.g. *Rattus, Apodemus, Microtus, Sorex* spp.). Larger mammals may also be parasitized, especially the predators of rodents, such as mustelids, fox and badger; and rarely, humans. *I. acuminatus* occasionally occurs on birds but we have had no records from dog (Keirans, 1984; Hillyard, 1996). The first observation of this species on dogs seems to be an accidental case. *I. acuminatus* can be infected with Bhanja and Uukuniemi viruses, *B. burgdorferi* s.l., *F. tularensis* and *C. burnetii* (Hillyard, 1996).

The single specimen of *D. marginatus* collected from dogs indicates that it infests this host only occasionally in Hungary. The seven specimens of this species which were collected on a sheep run in the vicinity of Nyíregyháza shows that it is still part of the Hungarian hard tick fauna. *D. marginatus* is known to infest large domesticated or wild mammals (Hillyard, 1996). It was found to be the second most frequent species after *I. ricinus* in Hungary (Babos, 1965) however, it may be an artefact of the incorrect synonymisation with *D. reticulatus* (Földvári and Farkas, 2005b). *Rickettsia slovaca* has been detected in *D. marginatus* in France (Beati et al., 1993), but there is no data on the risk of canine infestation (Table 3). Concerning its human health importance, Lakos (1997) reported that *R. slovaca* is responsible for the spread of TIBOLA (Tick-borne lymphadenopathy), a novel infectious disease of humans in Hungary and other European countries. *R. slovaca* infections were also confirmed with molecular methods in patients from France and Hungary (Raoult et al., 2002).

No specimens of *R. sanguineus* have been found in Hungary. It has a great veterinary importance among dogs in several European countries. This species is able to transmit *B. canis canis, B. canis vogeli, B. gibsoni, E. canis* and *R. conorii* (Shaw et al., 2001; Parola, 2004) (Table 3). Because of the ability of this species to establish in a single kennel, it is possible that it could be establish after introducing from abroad, as it was into the UK (Fox and Sykes, 1985), Czech Republic (Cerny, 1989) and Germany (Dongus et al.; 1996). The appearance of *R. sanguineus* needs to be monitored in Hungary, because the tourism from and into the Mediterranean countries is increasing.

The geographical range of tick species is increasing because they are finding niches in different climatic conditions (Talleklint and Jaenson, 1998). Ticks can move between European countries easier recently, since the pets are also increasingly moving with their keepers. Glaser and Gothe (1998) conducted a survey among practicing veterinarians in Germany in order to estimate the extent of tourism with and import of dogs, and to determine the range and preference of the foreign countries involved. The survey covered the years from 1985 to 1995 and included 5240 dogs, of which 87.2% were born in Germany and 12.8% abroad. More than half of the dogs had been taken abroad at least once between 1985 and 1995. Of the 2894 dogs taken abroad, 66.7% travelled to Mediterranean countries and 1152 of these had additional travels to other countries as well. The spectrum of all countries travelled to was very broad, but many dogs were taken regularly, repeatedly and exclusively to Austria, Switzerland, Italy, Spain or France. Other countries were visited only once for the majority of dogs. The analysis of the annual survey data revealed a steady increase of dogs along on trips from Germany to other countries, rising from 31.1% in 1990 to 40.8% in 1994. In any of these years, always more than 56% of these dogs were taken to Mediterranean countries. In addition to dog tourism, adventure travel with a growth rate of 10 % per annum since 1985, is increasingly popular and now constitutes the largest growing segment of the leisure travel industry worldwide (Jensenius et al., 2005). Each year, an increasing number of travellers (often accompanied by their dogs) thus visit remote places where they participate in trekking, bush walks, safaris, camping and other out-door recreational or occupational activities. Since some of these new tourist destinations are also important biotopes for ticks, a continuing increase of tick-associated diseases should be anticipated in the near future (Jensenius et al., 2005).

4.2. Tick-borne pathogens of dogs

4.2.1. Materials and methods

4.2.1.1. Detection of small canine piroplasms: case studies

In February 2002 a 6-month-old male Scottish terrier (dog 1) was taken to a local small animal clinic because of weakness, lethargy and anorexia. The owner had walked the dog in two different parks of Budapest about a week earlier. On clinical examination, the dog had pale mucous membranes and a rectal temperature of 39.5 °C. The animal was treated with antibiotics but no recovery was observed and abdominal pain was also detected on the following day. The veterinarian suspected that the clinical signs might be due to ileus, therefore the animal was sent to the Department and Clinic of Surgery and Ophthalmology, Faculty of Veterinary Science, Szent István University, Budapest. After ultrasonography, laparatomy was performed. Severe internal haemorrhage in the abdominal cavity due to the rupture of the spleen was diagnosed. The dog was splenectomised and the splenic impression smears were prepared from the ruptured spleen after operation for parasitological examination.

Dog 2 was a 3-year-old male collie from Budapest, kept in the garden of a detached house and walked regularly in a neighbouring forest. In March 2002 the dog was found to be healthy during a clinical examination. A blood sample was taken for routine haematological and biochemical profiles. Haematological values showed slight erythropenia and leucopenia and mild thrombocytopenia. Thin blood smears were prepared for parasitological examination.

Smears were stained with modified Wright's stain (Diff-Quik) and examined with oil immersion (1000 x magnification). Morphometric studies were performed on the intracytoplasmic parasites found in red blood cells.

4.2.1.2. Molecular examination of babesiae in blood and tick samples

Between 2002 and 2005, veterinary practitioners from all over the country were asked to collect blood samples from dogs showing clinical signs of babesiosis (e.g. fever, weakness, lethargy, loss of appetite, haemoglobinuria). The veterinarians were also asked to prepare a thin blood smear from each sample, to fix them with methanol and to stain with Giemsa solution. Blood samples were taken into EDTA tubes and kept at -20 °C before transporting to the Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent István Univerity, Budapest. Seventy blood samples were involved into the molecular analysis. DNA was isolated from 200 µl

amounts of EDTA blood from each dog using QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Unfed (collected by dragging) and fed (collected from dogs) *D. reticulatus* females were chosen for molecular analysis of babesiae. Nine samples were made out of either individual or pooled unfed ticks comprising of 23 specimens from Vecsés, five from Tét and two from Balatonkeresztúr. There were 81 samples of fed ticks; each contained 1-6 specimens. These ticks originated from 48 (either healthy or *Babesia*-infected) dogs from different geographical areas of Hungary. Tick specimens kept in 70% ethanol were washed in detergent, distilled water and then in phosphate buffer saline (PBS). In case of pooled samples, mixing of specimens from different locations was avoided. Both individual and pooled samples were homogenised separately in 100 µl PBS with sterilised small scissors in a microcentrifuge tube. DNA was isolated using QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. An overnight digestion in Proteinase K was performed.

The primers PIRO-A1 (5' AGGGAGCCTGAGAGACGGCTACC 3') and PIRO-B (5' TTAAATACGAATGCCCCCAAC 3') were used to amplify an approximately 450 bp region of the 18S rRNA gene. The forward primer, PIRO-A1 was developed by Muhlnickel et al. (2002) to amplify most *Babesia* species using sequence information from GenBank®. The reverse primer, PIRO-B, has been described previously by Olmeda et al. (1997). Two µl of extracted DNA was added to a 48 µl reaction mixture comprised of 1.5 units of Taq DNA polymerase (Promega, Madison, WI, USA), 200 µM of each dNTP, 25 pmol of each primer and 5 µl 10X PCR buffer and 1.5 mM $MgCl_2$ (Promega, Madison, WI, USA). Sterile destilled water was used as a negative control and positive controls were provided by Martin J. Kenny (Acarus Laboratory, Veterinary School, University of Bristol). Amplification was performed using a Tpersonal 48 thermal cycler (Biometra GmbH, Göttingen, Germany). An initial denaturation step at 94 °C for 10 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Final extension was done at 72 °C for 5 min followed by a hold step at 4 °C. Amplified DNA was subjected to electrophoresis in a 1.5% agarose gel (120 V, 40 min), pre-stained with ethidium-bromide and viewed under ultra-violet light.

Standard precautions to avoid DNA contamination (laminar flow hoods, separated work areas for reaction mixture preparation, DNA extraction, amplification and analysis of PCR products, including positive and negative controls etc.) were used in our laboratory to prevent carry-over of amplified products.

PCR products from blood samples and from ticks were chosen randomly for sequencing. After purification with Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA), ABI Prism® Big Dye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems

Division, Foster City, CA, USA) was used for DNA sequencing reactions. Samples were then examined using an ABI Prism® 3100 Genetic Analyser at the Agricultural Biotechnology Centre Gödöllő, Hungary. Sequences were checked with Chromas v.1.45 and compared to sequence data available from GenBank®, using the BLAST (Basic Local Alignment Search Tool) 2.2.10. program (http://www.ncbi.nlm.nih.gov/BLAST/) with the default settings. New sequences were submitted to GenBank® database.

4.2.1.3. Molecular examination of spirochetes in blood and tick samples

For molecular biological detection of spirochetes, 15 canine blood samples were selected randomly or because of *Borrelia* seropositivity (*Borrelia* automated ELISA Kit, Vidas, bioMerieux, USA) diagnosed by a veterinary diagnostic laboratory. Storage and transportation of samples and DNA extraction were carried out with the same method described for babesiae.

Unfed (collected by dragging) and fed (collected from dogs) female *I. ricinus* specimens were chosen for molecular analysis. Five samples were made out of unfed individual ticks: four from Balatonkeresztúr and one from Rádiháza. Thirty-three samples were made out of 1-12 fed tick specimens per sample. Fed ticks originated from several geographical areas of Hungary.

The primers BSL-F (5' AATAGGTCTAATAATAGCCTTAATAGC 3') and BSL-R (5' CTAGTGTTTTGCCATCTTCTTTGAAAA 3') were used, which amplify an approximately 250 bp region of the outer surface protein (osp) A gene from all Lyme disease spirochetes (Demaerschalck et al., 1995). Two µl of extracted DNA was added to a 20 µl reaction mixture comprised of 1.0 units of HotStartTaq DNA polymerase, 200 µM of each dNTP, 25 pmol of each primer and 1.5 mM $MgCl_2$ (HotStartTaq Master Mix, QIAGEN, Hilden, Germany). Sterile destilled water was used as a negative control and positive controls were provided by Martin J. Kenny (Acarus Laboratory, Veterinary School, University of Bristol). An initial denaturation step at 94 °C for 15 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. Final extension was done at 72 °C for 5 min followed by a hold step at 4 °C. Electrophoresis and of PCR products and sequencing from the unfed and fed tick samples were carried out with the same methods as with babesiae.

4.2.2. Results

4.2.2.1. Detection of small canine piroplasms

Many erythrocytes in the splenic impression of dog 1 were multiply infected with small parasites which appeared round to ring shaped, oval or comma-like. The infected red cells contained 1, 2, 4 or 8 organisms, but the parasites were not connected to each other (Fig. 36). The average diameter of parasites measured at random (n=35) was 1.81±0.34 µm. One tick specimen was removed from the animal and identified as a partly engorged female *D. reticulatus*. After dissection followed by methanol fixation and staining with Giemsa, no piroplasms were found in the smear prepared from the intestinal content of the tick.

After operation dog 1 was treated with imidocarb diproprionate (Imizol, Schering-Plough) at 5 mg/kg and antibiotics via subcutaneous injection and was then sent home. Two weeks later no clinical signs of the protozoal disease were observed during the next clinical examination of the dog. No blood sample was taken. Four months after operation the animal was found to be healthy.

Some erythrocytes of dog 2 contained comma-like small parasites which occurred singly or in pairs in a single cell (Fig. 37). The average diameter of parasites measured at random (n=35) was 1.72±0.39 µm. The dog was treated once with imidocarb diproprionate (Imizol, Schering-Plough) at 5 mg/kg via subcutaneous injection and was sent home. A month later no clinical signs of babesiosis were observed and no parasites were found in stained blood smears of the animal.

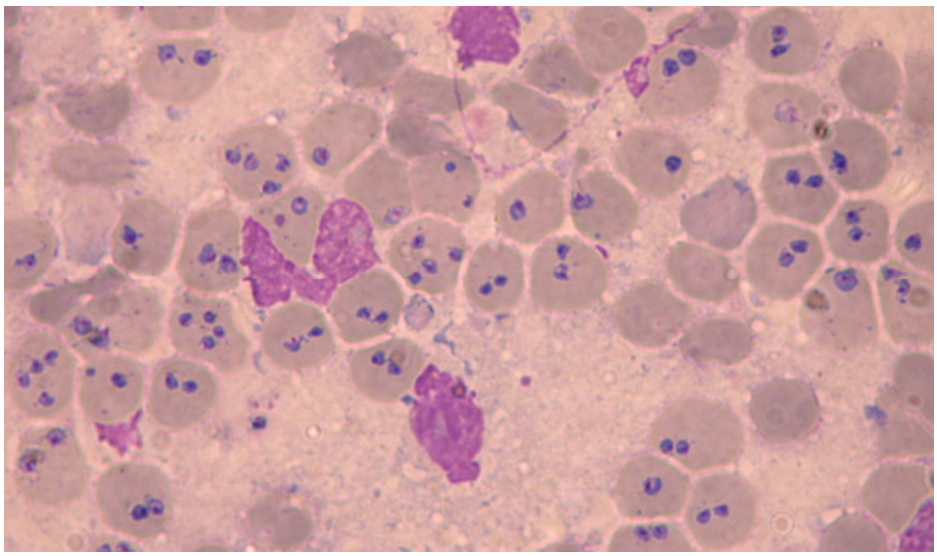

Figure 36. Splenic impression smear of dog 1 stained with modified Wright's stain (Diff-Quik) demonstrating severe parasitaemia. Multiply-infected erythrocytes with small, round to ring shaped, oval or comma-like parasites can be seen.

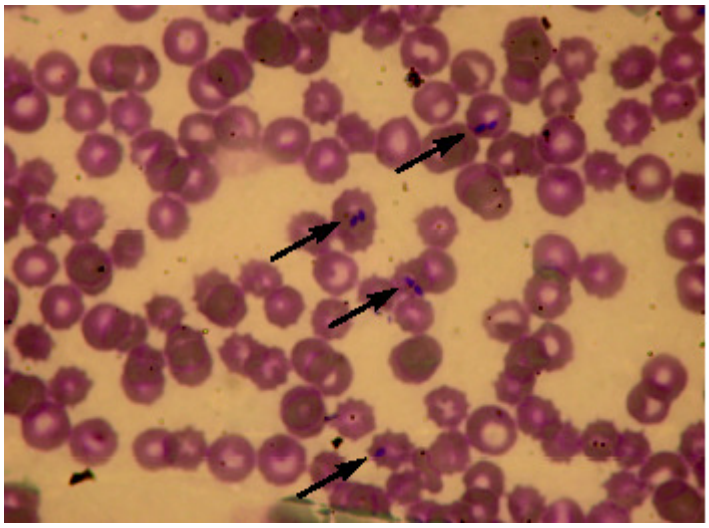

Figure 37. Thin blood smears of dog 2 stained with modified Wright's stain (Diff-Quik). Some erythrocytes contain small parasites which occur single or in pairs in a single cell.

4.2.2.2. Molecular examination of babesiae in blood and tick samples

Blood samples were collected from 70 dogs having clinical signs of babesiosis, but only 22 (31.4%) of these were sent to the department with usable stained blood smears. In all of these smears 4-5 µm long, single or paired pyriform intraerythrocytic parasites, characteristic for *B. canis* were observed using light microscopy.

An approximately 450 bp PCR product (Fig. 38) could be amplified from the blood samples of 60 (85.7%) dogs. Twenty-one positive samples originated from 10 districts of Budapest and 39 from 23 other locations of Hungary (Fig. 39). The sequences of sixteen randomly selected PCR products showed 100 % homology to one another or differed by 1-3 nucleotide which may represent sequencing error (being close to the primers) or minor variation. BLAST search against GenBank® revealed the highest similarity (99.3 to 100 %) with 18S rDNA partial sequence of *B. canis canis* (Table 2). From the 10 samples (14.3%) where no PCR products were detected, no blood smears were available.

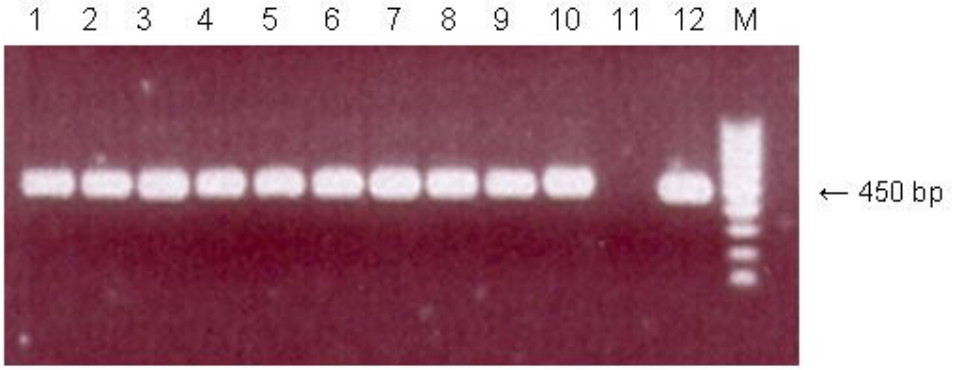

Figure 38. Ethidium bromide-stained 1.5% agarose gel showing amplification of a 450 bp product for *Babesia*-positive samples (molecular marker (M); *Babesia*-positive samples (1-10); negative control (11); positive control(12)).

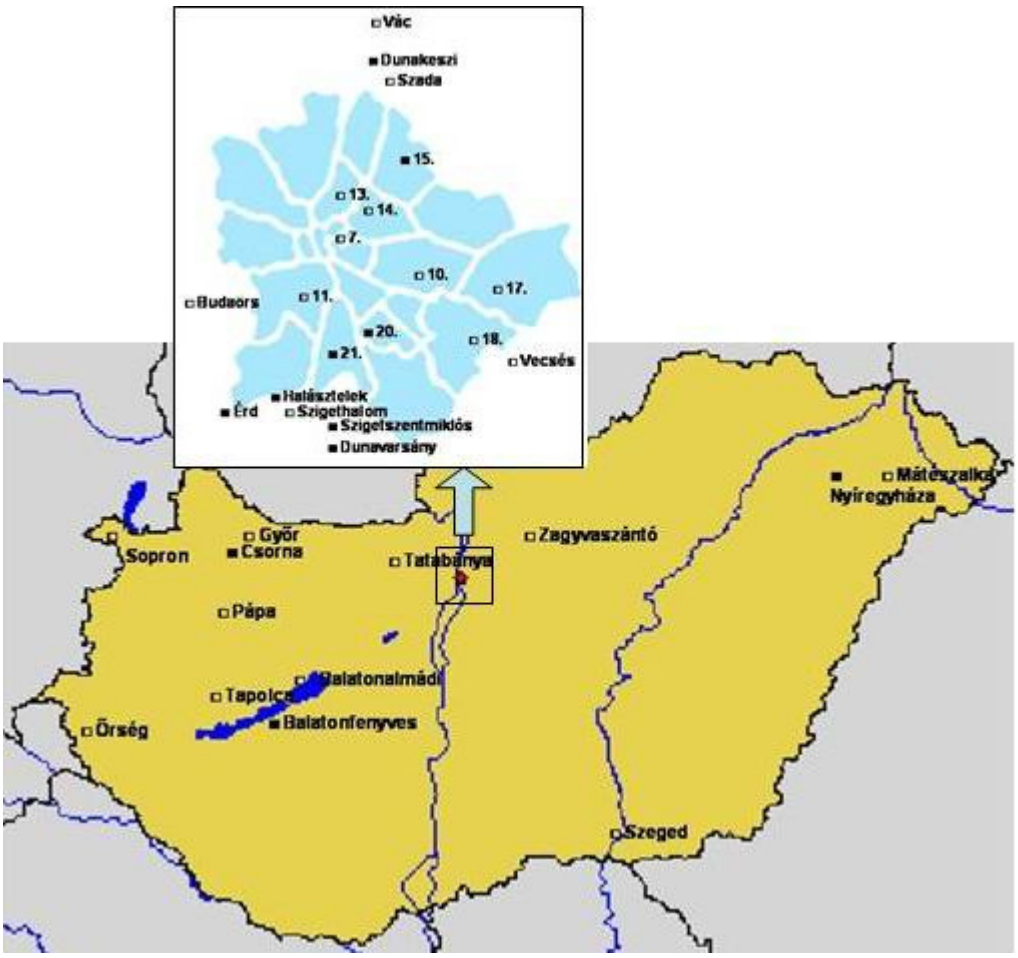

Figure 39. Origin of blood samples positive for *Babesia*-PCR (Budapest region in higher magnification; □ for PCR product, ■ for sequenced PCR product). District numbers within Budapest are indicated.

Table 2. Data of *Babesia* sequences from blood samples submitted to the GenBank® database.

Origin of sample	Accession Nr	Sequence length (bp)	Similarity to *B. canis canis* (%)
Budapest (15th district)	AY611729	405	100
Budapest (20th district)	DQ174279	414	100
Budapest (21st district)	DQ174280	404	100
Budapest (21st district)	DQ174281	405	100
Budapest	DQ174282	453	100
Halásztelek	AY611730	411	100
Dunakeszi	AY611731	412	99.8
Szigetszentmiklós	AY611732	411	99.8
Dunavarsány	AY611733	412	99.8
Érd	DQ174283	411	100
Balatonfenyves	DQ174284	424	100
Csorna	DQ174285	413	100
Csorna	DQ174286	411	100
Csorna	DQ174287	416	100
Csorna	DQ174288	415	100
Nyíregyháza	DQ174289	422	99.3

Six out of nine unfed *D. reticulatus* samples were PCR-positive for *Babesia* sp. Tick specimens infected with piroplasms originated from Tét and Balatonkeresztúr. Sequencing was not carried out on these PCR products.

An approximately 450 bp PCR product could be amplified from 37 (45.7%) of 81 samples containing fed *D. reticulatus* specimens. *Babesia* DNA was detected in 11 fed tick samples originating from Budapest and in 26 from six other locations of Hungary (Fig. 40). Among the 48 dogs from which *D. reticulatus* specimens were checked for *Babesia* by PCR, three showed only clinical signs of babesiosis. No blood sample was available from these animals but ticks collected from them were found to be PCR-positive. Ten dogs were PCR-positive for *Babesia* but in ticks removed from three of these animals, no *Babesia* DNA could be detected.

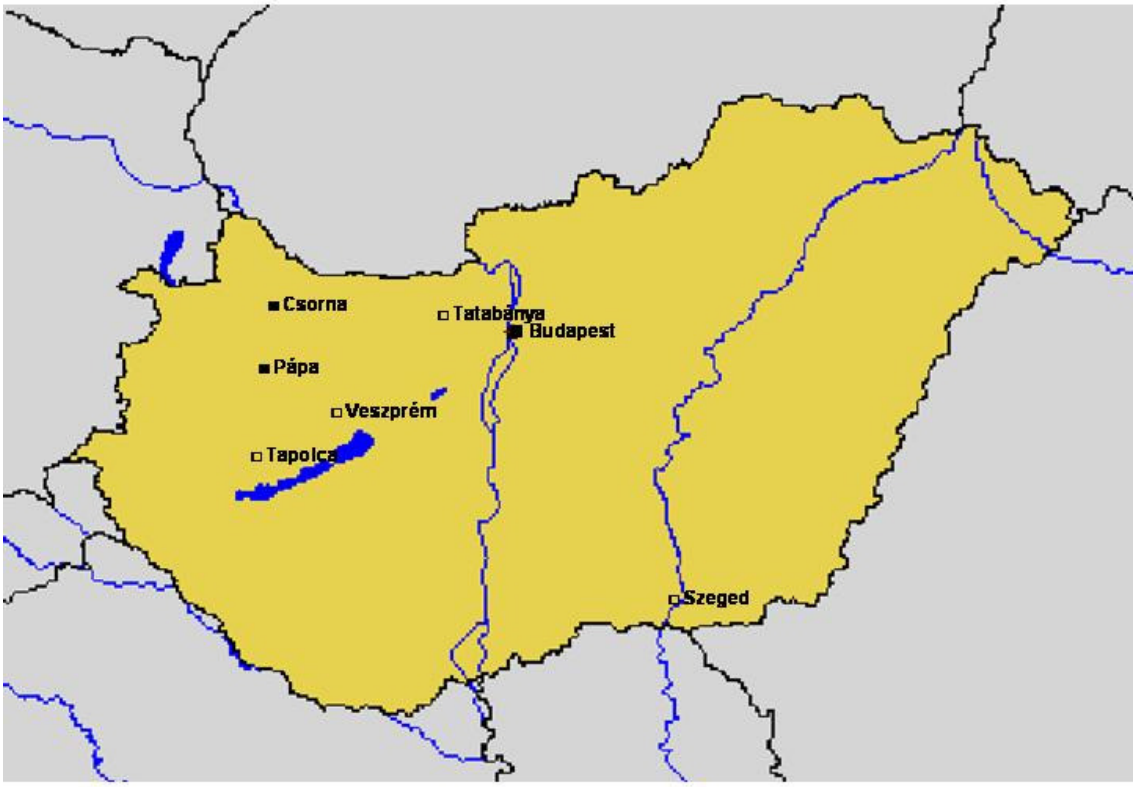

Figure 40. Origin of fed *D. reticulatus* samples positive for *Babesia*-PCR (□ for PCR product, ■ for sequenced PCR product).

Five PCR products were selected randomly for sequencing from the fed *D. reticulatus* samples. Sequences showed 100 % homology to one another or differed by 1 nucleotide which may represent sequencing error (being close to the primers) or minor variation. BLAST search against GenBank® revealed the highest similarity (99.8 to 100 %) with 18S rDNA partial sequence of *B. canis canis* (Table 3).

Table 3. Data of *Babesia* sequences from *D. reticulatus* females submitted to the GenBank® database.

Origin of sample	**Accession Nr**	**Sequence length (bp)**	**Similarity to *B. canis canis* (%)**
Budapest (20th district)	DQ181652	411	99.8
Budapest	DQ181653	412	100
Csorna	DQ181654	391	100
Pápa	DQ181655	413	100
Pápa	DQ181656	415	100

4.2.2.3. Molecular examination of spirochetes in blood and tick samples

No *Borrelia*-specific PCR product could be amplified in the 15 examined blood samples.

An approximately 250 bp PCR product could be detected in two (out of five) unfed *I. ricinus* samples originating from Balatonkeresztúr. Both of them were sequenced and they showed 100% homology to *Borrelia burgdorferi* s.s. sequences deposited in GenBank® (Table 4).

Five (15.2%) of 33 fed *I. ricinus* samples were PCR-positive. These engorged specimens containing *Borrelia* DNA were removed from dogs living in five different areas of the country (Fig. 41). Sequencing results and BLAST search for the three randomly chosen samples revealed the following: 100% homology with *B. afzelii* (Balatonfenyves and Mohács) and 100% homology with *B. garinii* (Pápa) (Table 4).

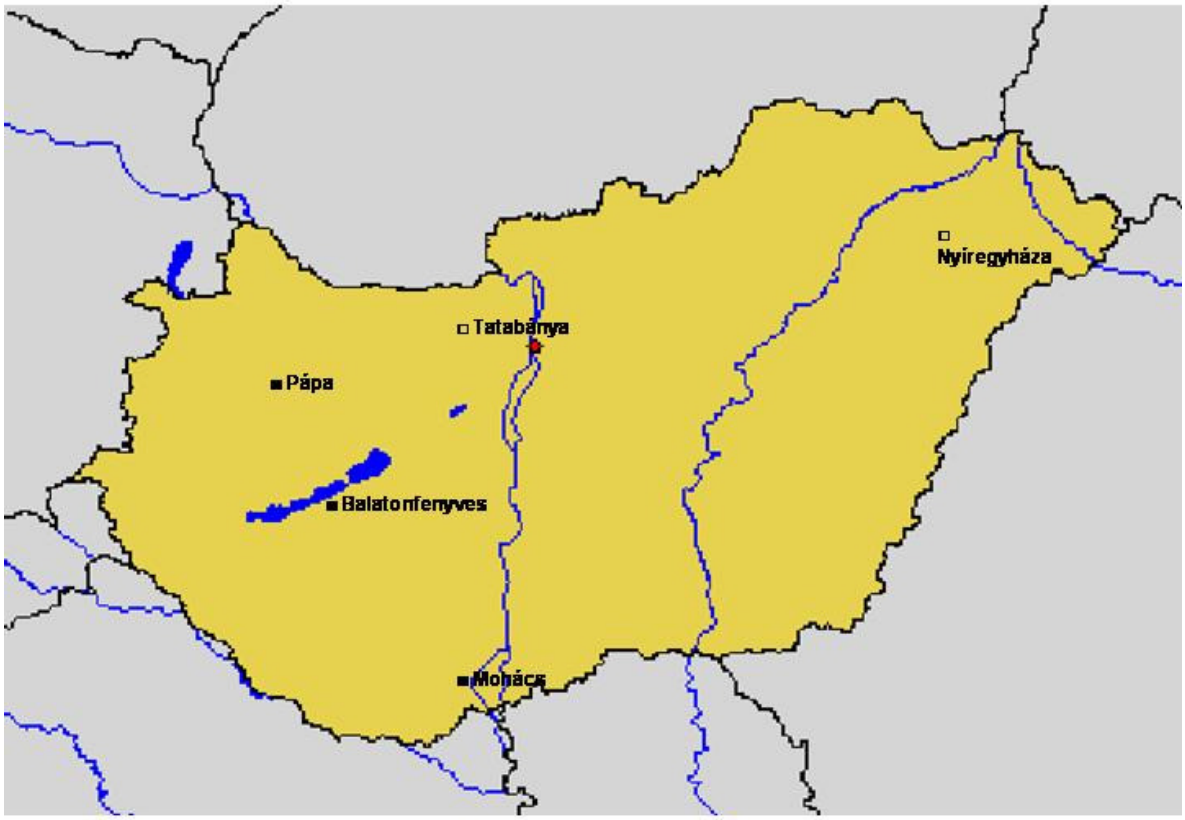

Figure 41. Origin of fed *I. ricinus* samples positive for *Borrelia*-PCR (□ for PCR product, ■ for sequenced PCR product).

Table 4. Data of *Borrelia* sequences from *I. ricinus* females submitted to the GenBank® database.

Sample	Origin of sample	Accession Nr	Sequence length (bp)	Similarity to *Borrelia genospecies*
Unfed *I. ricinus*	Balatonkeresztúr	DQ193524	258	100% *B. burgdorferi* s.s.
Unfed *I. ricinus*	Balatonkeresztúr	DQ193525	255	100% *B. burgdorferi* s.s.
Fed *I. ricinus*	Balatonfenyves	DQ193521	257	100% *B. afzelii*
Fed *I. ricinus*	Mohács	DQ193522	254	100% *B. afzelii*
Fed *I. ricinus*	Pápa	DQ193523	254	100% *B. garinii*

4.2.3. Discussion

4.2.3.1. Detection of small canine piroplasms

On the basis of the size of the intracellular parasites observed in both dogs, it was assumed that the animals were infected with small babesiae. In Hungary this was the first description of the presence of these parasites in dogs. The most frequently reported clinical signs of canine babesiosis, such as fever, anaemia, icterus and haemoglobinuria, were not observed in the infected animals. Babesiosis without a characteristic clinical picture is, however, not rare, because the symptoms can vary greatly depending upon the species and strain of *Babesia* and its virulence, the age of the animal, the stage of the disease and the complications caused by other pathogens (Kontos and Koutinas, 1997). It can be assumed that the immune response of these animals to these haemoprotozoa might also influence the clinical picture. This hypothesis is supported by the observation of rapid proliferation and high (10-13%) parasitaemia of *B. gibsoni* in a severe combined immune deficiency mouse model (Fukumoto et al., 2000).

Many infected erythrocytes were found in splenic impression smears prepared from the ruptured spleen of dog 1. These results are consistent with the observation by Schetters et al. (1998) who reported that the babesiae were found in the spleen of experimentally infected dogs. This internal organ increased considerably in size and was packed with infected erythrocytes. Multiple infections of many red blood cells suggested the proliferation of parasites.

Based on the identification of intraerythrocytic parasites on thin blood smears under oil immersion, it could not be confirmed whether *B. gibsoni* or other small babesiae caused the infection of the dogs. The occurrence of canine babesiosis caused by *B. gibsoni* has been reported from some European countries (Casapulla et al., 1988; Criado-Fornelio et al., 2003a). However, the clinical picture was not consistent with infection caused by *B. gibsoni* (Yamane et al., 1993, Zahler et al., 2000b, Kocan et al., 2001). It might be that another species caused the infection of the two dogs because *B. gibsoni* is more pathogenic and more difficult to treat than the infections reported here. If dogs infected with *B. gibsoni* do not receive prompt, effective treatment they generally die (Yamane et al., 1993). Furthermore, *R. sanguineus*, the vector of *B. gibsoni* has never been reported to occur in the country (Babos, 1965, Földvári and Farkas, 2005a). It might be therefore possible that the infection of these two animals was caused by other small babesiae which have been reported from dogs as *B. gibsoni*-like or *B. microti*-like (later named as *T. annae*) in Europe (Zahler et al., 2000b,c, Camacho et al., 2001, 2003), but other unknown species of small babesiae cannot be excluded either. Considering the differences in the pathogenity of the two cases (rupture of the spleen in dog 1 and no clinical signs in dog 2), we cannot exclude that different pathogens were

involved in these two dogs. Because these parasites are morphologically not distinct enough to be identified definitively by light microscopy, a more specific diagnostic method such as DNA amplification by PCR and sequencing is needed. However, we could not carry out a molecular analysis, because, unfortunately, no blood or spleen samples were preserved from any of the two dogs.

Further research is needed to know the species, occurrence, vector and origin of small babesiae occurring in Hungary. They might have been introduced into the country by asymptomatic carrier dogs, either imported from abroad or returning from holidays in endemic countries. There is a great risk that infections caused by small babesiae can be easily spread in Hungary because of the abundance of ticks in many places in the country and the easy transportation of asymptomatic carrier dogs. The dogs of these cases, however, never travelled abroad.

Although a specimen of *D. reticulatus* was found on dog 1, we were unable to demonstrate a casual relationship between the tick and the *Babesia*-infection. Consideration should be given to assessing the vector competence of the local tick species, notably *D. reticulatus* and *I. ricinus* that feed on dogs in areas where canine babesiosis occurs (Farkas and Földvári, 2001). *I. hexagonus* which was found to be also common (Babos, 1965) and infests dogs (Földvári and Farkas, 2005a) in Hungary has been suspected to be the vector of *T. annae* in northern Spain (Camacho et al., 2003).

Babesiosis caused by small babesiae may pose a serious threat to dogs in Hungary because the clinical disease caused by either large or small *Babesia* species is often indistinguishable, serological diagnosis of the two forms is hampered by cross-reactivity and because not all the anti-*Babesia* drugs are effective against small babesiae (Yamane et al., 1993, 1994). For these reasons it is crucial to properly diagnose canine babesiosis and adequately identify the causative agents with molecular methods in the future. The gathering of data on the epidemiology of canine babesiosis and the education of veterinarians about the risks and methods of controlling small babesiae infections are, therefore, vital for the development of effective control programs. Molecular identification of the disease agent, data on its occurrence and on the vector would be required to determine whether these parasites could be dangerous for humans, especially for immune suppressed or splenectomised patients.

4.2.3.2. Molecular examination of babesiae in blood and tick samples

Babesiosis has been an endemic disease among dogs in Hungary for many decades (Wetzl, 1905; Miklósi, 1931,1932; Horváth and Papp, 1974; Horváth and Papp, 1996; Csikós et al., 2001). It has been demonstrated with traditional methods (e.g. size of the intraerythrocytic forms and

experimental transmissions) that the causative agent is *B. canis* (syn. *Piroplasma canis*) (Wetzl, 1905) and its vector tick species is *D. reticulatus* (syn. *D. pictus*) (Janisch, 1986). Our present work represents the first molecular survey on canine babesiosis in the country attempting to identify and characterize the subspecies (genotype) of this large canine piroplasm.

Polymerase chain reactions with the piroplasm-specific primers, PIRO-A1/PIRO-B were positive for 60 out of 70 samples sent to our department for *Babesia* analysis. PCR-negative samples (n=10) originated from those dogs in which babesiosis was diagnosed only by clinical examination but not tested by blood smear. These findings point out that diagnosis of canine babesiosis can not solely be based on clinical symptoms. On the other hand, there can be cases, when samples found negative by microscopic examination of blood smears, still can turn out to be positive by the considerably more sensitive PCR methods (Jefferies et al., 2003; Birkenheuer et al., 2003; Matjila et al., 2005).

Geographical origin of PCR-positive samples proved the presence of piroplasms (and so the infected tick vectors) in many districts of Budapest and in several other parts of the country including north-eastern (Nyíregyháza, Mátészalka) and south-eastern (Szeged) regions, from where no babesiosis had been reported earlier (Horváth and Papp, 1996). The occurrence of canine babesiosis in these parts of Hungary is in accordance with the geographical distribution of the vector, *D. reticulatus* (Földvári and Farkas, 2005a), and suggests that larger part of the country is endemic for this tick-borne disease than it has been thought (Földvári and Farkas, 2005b).

Sixteen PCR products from blood samples were chosen randomly for sequencing, and showed 99 to 100% similarity with the *B. canis canis* sequences deposited in GenBank®. This is in accordance with our prediction based on previous information on the vector species (Janisch, 1986; Farkas and Földvári, 2001; Földvári and Farkas, 2005a). Our present work provides the first evidence concerning the subspecies (genotype) of *B. canis* which has caused severe disease among dogs in Hungary.

We provided molecular biological evidence for the presence of babesiae in unfed *D. reticulatus* specimens for the first time in Hungary. PCR-positive specimens originated from Tét and Balatonkeresztúr, two areas where we have information on *Babesia* infection of dogs (Földvári et al., 2005; Földvári and Farkas, 2005b). Because of geographical, epidemiological and biological (vector-parasite specificity) reasons, we suspect that these PCR products most probably reflect the presence of *B. canis canis*. However, sequencing and further studies on the prevalence of *Babesia* sp. in free living *Dermacentor* sp. is needed.

During molecular examination of 81 tick samples originating from 48 dogs, we detected *Babesia* DNA from engorged *D. reticulatus* specimens for the first time in Hungary. Nearly the half (37; 45.7%) of the samples containing fed females were positive for *Babesia*-specific PCR. The

broad geographical distribution of the positive samples is in accordance with our findings of the occurrence of *Babesia*-positive canine blood samples (Földvári et al., 2005; Földvári and Farkas, 2005b). Three dogs which had been diagnosed merely on having clinical signs of babesiosis had *Babesia*-infected ticks. Ten dogs had PCR-positive blood samples, however, we could find infected ticks only from seven of them. This indicates that a *Babesia*-infected dog does not necessarily harbour infected *D. reticulatus* ticks on itself. The infection of these dogs could have happened earlier or by another tick specimen.

On the other hand, dogs infested with *Babesia*-positive *D. reticulatus* specimens are not necessarily clinically ill or infected with piroplasms (unpublished data). Since it is assumed that an infection with the parasites usually happens after 2-3 days of tick attachment (Beugnet, 2002), it is possible to detect infected ticks on a non-infected dog within the first days of the tick bite. In addition, an immune defence of dogs against *Babesia* infection also has to be taken into consideration. Brandão et al. (2003) examined the immunological difference between *B. canis*-infected dogs treated and not treated with antibabesial drug. The use of imidocarb diproprionate in two doses of 7 mg/kg, with an interval of 14 days, seemed to be effective in eliminating canine babesiosis infection leading to clinical improvement and restoration of normal laboratory values. However, they also observed that treatment resulted in decreased antibody titers, which might make dogs more susceptible to reinfection in a short period of time. The untreated dogs were able to develop an effective immune response with a longer maintenance of antibody titers that protected them against a challenge with an homologous *B. canis* strain. The fall in antibody titers in untreated dogs suggests that there was a natural clearance of infection. This means that protection is not solely reached by the premunition state, but it could also be due to the constant antigenic stimulation produced by periodic exposure of dogs to the infectious agent by tick bites. Thus, it is very probable that untreated animals are more resistant to an homologous challenge infection (Lewis et al., 1995; Penzhorn et al., 1995; Schetters et al., 1997b).

The five PCR products which have been sequenced provided molecular evidence for the presence of *B. canis canis* in engorged *D. reticulatus* specimens in Hungary. Together with the 16 sequences obtained from blood samples, we can assume that the genetic variance of the partial 18S rDNA that has been amplified is rather small among the Hungarian large canine piroplasms. All the examined parasites were homologous to or very similar with *B. canis canis* sequences previously submitted to GenBank®. This can be explained epidemiologically, since in Hungary there is no vector species for the other large canine *Babesia* commonly occurring in Europe, namely *B. canis vogeli*. The presence of this subspecies can only be expected when *R. sanguineus*, the vector is present in an area, like in France or Slovenia (Cacció et al., 2002; Duh et al., 2004).

The scarce information concerning clinical and immunological aspects of infections with

different subspecies of *B. canis* (Schetters et al., 1997a) indicates, that the frequently used *diagnosis ex juvantibus* (i.e. diagnosis based on the recovery of dogs following antibabesial treatment) is not recommended. Furthermore, infections with other piroplasms, like *B. canis vogeli* (proved to be present in France by Cacciò et al., (2002) and in Slovenia by Duh et al., 2004)), *B. canis presentii* (recently observed as a new subspecies in cats from Portugal, Spain and Israel by Criado-Fornelio et al. (2003c) and Baneth et al. (2004)) or the novel unknown large *Babesia* sp. (Birkenheuer et al., 2004) cannot be excluded, especially in case of a previous visit in endemic areas. Molecular biology provides a powerful method not only in subspecies (genotype) identification, but also in cases when symptoms and/or blood smears do not provide definitive diagnostic information for the veterinarian. For these reasons, it can be also diagnostically important to determine the species, subspecies and genotype that causes canine babesiosis.

4.2.3.3. Molecular examination of spirochetes in blood and tick samples

Some researchers have suggested a close association between population/distribution of ixodid ticks and Lyme disease prevalence in humans and dogs (Lissman et al., 1984; Magnarelli et al., 1987). Lyme borreliosis occurs frequently in Hungarian human population (Lakos, 1991), and it is reported that the number of *Borrelia* seropositive dogs is increasing (personal communication). In addition, *I. ricinus*, the most important vector species of *B. burgdorferi* s.l. has been common in the country (Janisch, 1959; Babos, 1965, Farkas and Földvári, 2001) both on dogs and in the field collections. Although Lakos et al. (1991) found B. *burgdorferi* s.l. in unfed ticks from Hungary, no direct evidence on the genospecies of the spirochetes of dogs or ticks has been published yet. Therefore, we searched for the presence of borreliae in *I. ricinus* ticks collected from field and in canine blood samples. Three methods are generally used for the detection of *B. burgdorferi* s.l. in blood and tick samples: microscopy (dark field, phase contrast, Giemsa-stained smears, direct and indirect immunofluorescence), cultivation (in Barbour-Stoenner-Kelly medium), and polymerase chain reaction (PCR) for spirochetal DNA. Cultivation is the least sensitive of these techniques and PCR is the most sensitive method (Hubalek and Halouzka, 1998). To reach a high specificity, we combined PCR with sequencing which enables the genospecies determination of the PCR-positive samples.

The 15 canine blood samples involved in our preliminary study were PCR-negative for *Borrelia* sp. Ten were chosen because DNA have already been extracted from them (examining for the presence of piroplasms, all were *Babesia* PCR-positive) and five of them were taken from dogs with a serodiagnosis of borreliosis. Although there are cases (like early phase of the disease) when PCR detection in blood samples is useful (Skotarczak and Wodecka, 2005), spirochetes tend to

migrate to different parts of the body (skin, fascia, joints) where they can persist (Chang et al., 1996). This can be an explanation for the absence of *Borrelia* DNA in the blood of seropositive animals. Another reason may be that a serologically positive result can also reflect a previous infection with the spirochete which cannot be found in the blood any more (Levy and Magnarelli, 1992). Hovius et al. (1999b) examined the presence of *B. burgdorferi* s.l. in different organs of symptomatic and asymptomatic dogs in the Netherlands with molecular methods. They detected *B. burgdorferi* s.s., *B. garinii*, *B. afzelii* and *B. valaisiana*. Symptomatic dogs showed slightly higher prevalence of *Borrelia* in liver samples (9 of 15) than asymptomatic dogs (9 of 23). *B. garinii* was the most prevalent species and occurred together with up to three other species in one liver sample. *B. burgdorferi* s.s. however, was predominantly detected in samples of synovial membranes, skin, cerebrospinal fluid, bladder, heart, and bone marrow. Nine out of 10 symptomatic dogs with a very high antibody titre were PCR-positive for *Borrelia* in one or more of these tissues. They concluded that dissemination in naturally infected European dogs occurred and that the two most prevalent species, *B. burgdorferi* s.s. and *B. garinii*, differ in their tropism.

Two of the five samples containing questing females from Balatonkeresztúr were PCR-positive for *Borrelia* sp. During sequence analysis, both spirochetes were found to be identical to *B. burgdorferi* s.s. This species is commonly found in human patients and has recently been detected in Dutch (Hovius et al., 1999b) and Polish (Skotorczak and Wodecka, 2005) dogs. Although Lakos et al. (1991) has already provided evidence for the presence of spirochetes in unfed *I. ricinus* specimens, our present work could identify the genospecies for the first time in Hungary.

Five of the 33 samples containing engorged *I. ricinus* females were found to be PCR-positive for *Borrelia* sp. which provides the first molecular detection of the spirochetes in ticks removed from dogs in Hungary. All five positive samples originated from different parts of the country including Transdanube area and Nyírség. Although only three samples were sequenced, we found two different genospecies. Two were identical to *B. afzelii* and one was homologous with *B. garinii*. Both of these were described in human (Michel et al., 2003) and dogs (Hovius et al., 1999b). We had neither blood samples from these animals nor data on a possible *I. ricinus* infestation earlier. However, no clinical signs of borreliosis were detected in dogs carrying *Borrelia*-infected ticks. This can mean an infection with no clinical signs, a very early phase of infection or no infection at all. It is important to note that infection with *B. burgdorferi* s.l. only rarely leads to clinical symptoms in dogs (Shaw et al., 2001).

Hovius et al. (1998) determined the prevalence of *Borrelia* species by PCR in 138 ticks collected from dogs which were walked near the city of Eindhoven, the Netherlands. The PCR amplified the spacer region between the 5S and 23 S rRNA genes, and the *Borrelia* species was identified by hybridization with specific probes. *B. burgdorferi* s.l. was present in 20 of 138

(14.5%) ticks. Four species were identified: *B. burgdorferi* s.s. (n=8), *B. afzelii* (n=4), *B. garinii* (n=2), and *B. valaisiana* (n=2). Three ticks contained more than one species, all including *B. burgdorferi* s.s., and one tick even contained four species. Further investigation is needed to get more information on the prevalence of the Lyme disease spirochetes in Hungary both in dogs and in ticks removed from them.

Kurtenbach et al. (2002) reviewed the host association of *B. burgdorferi* s.l. and divided it into at least three ecological groups: (1) genospecies that are adapted to small mammals; (2) genospecies that are adapted to birds; and (3) genospecies that are not specialized (Fig. 42). The Hungarian tick isolates represent different groups: *B. burgdorferi* s.s. belongs to the unspecialized, *B. afzelii* to the rodent and *B. garinii* to either the bird or the rodent type.

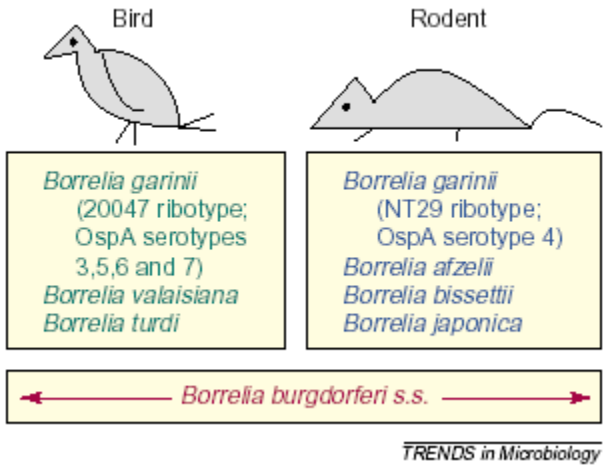

Figure 42. Schematic diagram of host specificity of *B. burgdorferi* s.l. (Kurtenbach et al., 2002.)

5. NEW RESULTS AND CONCLUSIONS

In the course of our studies on ticks and tick-borne diseases of dogs in Hungary the following new results have been achieved:

- A figured practical identification key has been designed for the sixteen hard tick species which occur on dogs in Europe.
- In 29 veterinary clinics from six districts of Budapest and 13 counties, 1779 tick specimens were collected from 606 dogs. Most hosts were usually infested with a single female and very few of them had many ticks. The most preferred sites of tick attachment in decreasing order were head, neck and legs. *Ixodes ricinus* and *Dermacentor reticulatus* were the most common species. *Ixodes canisuga, Haemaphysalis concinna, Ixodes hexagonus, Ixodes acuminatus* and *Dermacentor marginatus* were also found. New data have been provided about the geographical distribution of *Dermacentor reticulatus*, because the specimens of this species were collected in north-eastern and south-eastern parts of the country too where they had not been found before.
- Field collections in 31 locations provided new data on the geographical and seasonal occurrence of *I. ricinus, D. reticulatus* and other tick species as well.
- The occurrence of small canine piroplasms in two dogs was described for the first time in Hungary. These were autochtonous infestations but we need further investigations to know the species, occurrence, vector and origin of this pathogen.
- The subspecies *Babesia canis canis* was identified to be the causative agent of babesiosis caused by large *Babesia* sp. in dogs using molecular biological methods. It was also proven with molecular methods that the geographical distribution of canine babesiosis is larger in the country than it has been previously known.
- *Babesia* DNA was detected in free-living and engorged *D. reticulatus* females for the first time in the country. Presence of *B. canis canis* in engorged *D. reticulatus* specimens removed from dogs was also demonstrated with molecular methods.
- Molecular evidence was found for the presence of *Borrelia* sp. in free-living and engorged *I. ricinus* females for the first time in Hungary. Three species, *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* were identified with sequence analysis which are pathogenic to both dogs and humans.

The presence of dogs around humans increases the number of their ectoparasites in the houses, flats and surroundings (Shaw et al., 2001). An increase in the number of ticks in the vicinity of humans increases the risk of a human tick infestation. So, dogs can be considered as reservoirs of ticks in human environments posing the risk of new and re-emerging infectious diseases. Due to climatic changes towards global warming, imported tick species may adapt to new area and might be considered as epidemiological markers for a number of infectious agents transmitted by them (Kenny et al, 2004b). This is what makes monitoring, mapping and identification of ixodid ticks necessary in the future.

Dogs in Hungary were found to be infested with species of ticks that included competent disease vectors. With support of molecular biological methods (Sparagano et al., 1999; Monis et al. 2005), infections of various tick-borne parasites should be studied in both the vector and the host to clarify the epidemiological role of both the tick and the dog in the transmission of these pathogens. In order to gain more information about the occurrence of hard ticks and their epidemiological and epizootiological role, we plan to continue this study with increased number of field collection sites and veterinary clinics in new areas. Deepening our understanding of the ecological needs of the tick species found on dogs in Hungary would further enhance the prevention of tick-borne diseases in Europe.

Several factors have probably contributed to the emergence of canine babesiosis and other tick-borne diseases in Europe. They include the increase in outdoor activities and travels among Europeans resulting in an increased contact with ticks and an increased risk of transmitted diseases; the development of new techniques for the detection of organisms; the use of molecular methods for their characterization; as well the curiosity and awareness of clinicians about atypical cases (Parola and Raoult, 2001). Numbers of *Babesia* spp., *Rickettsia* spp., *Anaplasma* spp. and *Ehrlichia* spp. of unknown pathogenicity have been found in ticks and represent potential candidates for new human and/or canine tick-borne diseases to be described in the future. Finally, global climate changes could also have an influence on the epidemiology of tick-borne diseases in Europe (Lindgren et al., 2000). Indeed, it was recently suggested that increased average temperature during the winter would be responsible for an extension northern limit of the area of distribution of *I. ricinus*, as well as an increased population density of these ticks from 1980s to 1990s (Lindgren et al., 2000). Thus, this could lead to higher incidence of the diseases transmitted by *I. ricinus* (and potentially other species), as well as an increased risk area. For example, some authors hypothesized that this could explain the higher incidence of the tick-borne viral encephalitis transmitted by *I. ricinus* in Sweden (Lindgren and Gustafson, 2001). Although this hypothesis was largely discussed (Randolph and Rogers, 2000; Hay, 2001; Randolph, 2001; Randolph, 2002), we should keep in mind that climatic change is an other factor which could modify the epidemiology of both human and canine tick-

borne diseases and contribute to the emergence of new diseases in the future.

Further research is needed to know the origin of small babesiae occurring in Hungary. With the advances of molecular techniques we plan to characterize this parasite with PCR and sequencing. A targeted sampling method will be needed in the veterinary clinics to select the small *Babesia* infections from the relatively common *B. canis* infection.

For both veterinary and public health reasons, the study of *B. burgdorferi* s.l. in host seeking ticks became an urgent and necessary issue in Hungary. We intend to collaborate with Lyme disease specialists, human and animal diagnostic laboratories to launch further molecular surveys and gain more information about the reservoir species and the spatio-temporal distribution of Lyme disease spirochetes in the country.

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7. OWN PUBLICATIONS

7.1. In peer reviewed scientific journals

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7.2. Presentations on international conferences

Földvári, G., Farkas, R. 2004. Ixodid tick species attaching to dogs in Hungary. – poster at the 9th European Multicolloquium of Parasitology, Valencia, Spain

Földvári, G., Hell, É., Farkas, R. 2004. Occurrence of *Babesia canis canis* in Hungarian dogs. – lecture at the 9th European Multicolloquium of Parasitology, Valencia, Spain

Farkas, R., Beugnet, F., **Földvári, G.** 2005. Do you really think that acaricides could prevent babesiosis? - lecture at Merial 3rd Parasitology Dermatology Symposium, Paris, France

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