Borrelia burgdorferi sensu lato and Anaplasma phagocytophilum in the Czech Republic

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Abstract

The aim of this thesis was to assess the occurrence of two tick-borne bacteria, *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum*, in ticks, wild and domestic animals in the Czech Republic. In ticks, similar prevalence of both bacteria was observed. In rodents, the majority of infections were caused by *B. afzelii* while the infection with *B. burgdorferi* s. s. was also quite frequent. Infection with *B. burgdorferi* s. l. was more common in bank voles than in wood or yellow-necked mice. The prevalence of anti-*Borrelia* antibodies was higher in wood or yellow-necked mice than in bank voles. *A. phagocytophilum* was in a higher percentage of cases in the deer family and hares as compared to foxes and boars. We observed a similar prevalence of anaplasmosis in all domestic animals tested. We demonstrated that symptomatic dogs had a higher chance to be infected with *A. phagocytophilum* than asymptomatic dogs. Our findings suggest that the exposure to *B. burgdorferi* s. l. and *A. phagocytophilum* is common in vectors, reservoirs and hosts in the Czech Republic.

Molecular and serological techniques for detection of these pathogens are also described in this thesis, including conventional PCR, nested PCR, real-time PCR with DNA quantification and melting curve analysis, RFLP analysis of the 5S-23S rDNA intergenic spacer and direct sequencing of the 16S rDNA. We found that real-time PCR is a very fast method but it cannot distinguish within the group of *Borrelia* genospecies and that of *A. phagocytophilum* variants.

Finally, clinical signs and diagnostic findings for three examples cases of *Borrelia* infection in dogs are presented. We showed that borrelial infection must be considered not only in cases with febrile and orthopaedic signs but also for many other clinical syndromes.
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Chapter 1

Introduction

This thesis studies the occurrence of two tick-borne bacteria, *Borrelia burgdorferi* sensu lato (s. l.) and *Anaplasma phagocytophilum*, in ticks and wild and domestic animals in the Czech Republic. This contributes to the understanding of the natural cycle of these bacteria. So far only a limited data has been available in the Czech Republic, especially concerning animal hosts. We also describe the molecular and serological techniques for detection of these pathogens. Finally, we give clinical signs and diagnostic findings for three examples cases of *Borrelia* infection in dogs.

We will now describe these pathogens, their vectors and hosts, and the diseases they cause, mainly focusing on the situation in Europe.

1.1 Lyme borreliosis

Lyme borreliosis is a multi-organ disease of mammals in the northern hemisphere. The Lyme disease is named after Lyme, a town in Connecticut, USA, where the first cases of this disease were reported in 1975 (Steer et al. 1977). Before that, at the end of the 19th century, a chronic skin disorder had been described in Europe and was later named acrodermatitis chronica atropicans (ACA) (Buchwald 1883). At the beginning of the 20th century in Sweden, Dr. Afzelius presented a patient with skin lesion, which he called Erythema migrans (EM) (Afzelius 1910). It is known that in humans, both ACA and EM are among clinical symptoms of the Lyme disease. Other clinical symptoms include Borrelial lymphocytoma, neurological manifestations, joint manifestations, and cardiac manifestations. Lyme borreliosis is categorized as a zoonotic disease in humans because the infection is maintained in nature by animals. Humans are dead-end hosts and are not involved in natural circulation of *B. burgdorferi* s. l. However, humans can be infected and subsequently can fall ill (Humair and Gern 2000, Gern 2009).
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1.1.1 *Borrelia burgdorferi sensu lato*

Lyme disease agent was discovered by Willy Burgdorfer in North America in 1982 and was isolated and cultured from the midgut of *Ixodes ricinus* ticks, and from patients with Lyme disease in North America and in Europe (Burgdorfer et al. 1982, Barbour 1984). This spirochete was identified as a new species among *borreliae* and named *Borrelia burgdorferi* (Johnson et al. 1984). In 1998, human pathogens *Borrelia afzelii* and *Borrelia garinii* were described in Europe and Asia (Baranton et al. 1998) but there were not found in North America. *Borrelia burgdorferi* *s. l.* includes at least 15 genospecies, four of them are known to be pathogenic for humans: *Borrelia burgdorferi* sensu stricto (s. s.), *Borrelia afzelii*, *Borrelia garinii* and *Borrelia spielmanii* (Johnson et al. 1984, Baranton et al. 1992, Canica et al. 1993, Wang et al. 1999, Baranton and Martino 2009, Rudenko et al. 2009a, Rudenko et al. 2009b). Occasionally, *Borrelia valaisiana*, *Borrelia lusitaniae* and *Borrelia bissetii* were also detected in patients (Picken et al. 1996, Rijpkema et al. 1997, Collares-Pereira et al. 2004, Fingerle et al. 2008, Hulínská et al. 2009, Nau et al. 2009, Rudenko et al. 2009c).

*B. burgdorferi* *s. l.* has been reported in all Europe from at least 30 countries (Hubálek and Halouzka 1997, Hubálek 2009). Although there is much that is not yet known about the distribution of the various genospecies in Europe, current knowledge suggests that *B. garinii* and *B. afzelii* are the most frequent and most widely distributed species whereas *B. burgdorferi* *s. s.* is less frequent (Piesman and Gern 2004, Gern 2009).

*Borrelia* is a spirochete, a narrow, long, helical bacterium, measuring 15 to 30 µm in length by 0.2 to 0.3 µm in width. This spirochete has a fragile outer membrane surrounding the protoplasmic cylinder consisting of a peptidoglycan layer (Burgdorfer et al. 1982). *Borrelia* spp. circulate between ticks and a wide variety of vertebrates, including different mammals, some bird species and few reptiles (Humair and Gern 2000, Piesman and Gern 2004, Gern 2009).

1.1.2 Vectors

The main *Borrelia* vectors are ticks. A vector must be shown to be capable of getting infected and later infecting naive hosts. Ticks usually get infected by feeding as larvae, moult to the next life stage and then infect their next feeding host (Humair and Gern 2000, Gray et al. 2002).

The epidemiologically most important ticks transmitting *B. burgdorferi* *s. l.* to humans are *Ixodes persulcatus* (northern middle Asia), *Ixodes scapularis* (eastern North America), *Ixodes pacificus* (western North America) and *Ixodes ricinus* (Europe) (Parola and Raoult 2001). In Europe in particular, three tick species have been described as being vectors of *B. burgdorferi* *s. l.: I. ricinus, Ixodes hexagonus
and *Ixodes uriae*.

All are three tick stages (larva, nymph and adult female) feed on different hosts. Although adult male *Ixodes* sometimes ingest fluids from hosts, they do not ingest significant amounts of blood and their role as vectors is probably insignificant, however this has not been thoroughly evaluated (Piesman and Gern 2004). Tick vectors acquire *borrelia* while feeding on infected reservoir hosts or by cofeeding (Gern 2009). *Borrelia* remains inactive in the midguts of the ticks while they moult. In the next developmental stage the spirochetes disseminate rapidly to all other body organs, including salivary glands, wherefrom the *Borrelia* is transferred to vertebrates. If the *Borrelia* load in a tick is high, or for some *Borrelia* species, the spirochetes may be present in salivary glands and hence be transferred to the host even earlier (Gern 2009, Hubálek 2009). Transovarial transmission in ticks is rare (Humair and Gern 2000, Gray et al. 2002).

Ticks are dependent upon the availability of suitable host individuals for each life stage. They also need a microclimate with high relative humidity during the off-host phases. Host-seeking is a seasonal activity and is strongly dependent on environmental factors. Proliferation of *B. burgdorferi* s. l. requires the simultaneous occurrence of ticks and vertebrate populations capable of transmitting *borreliae* (Humair and Gern 2000, Gray et al. 2002).

The reported prevalence of *B. burgdorferi* s. l. in ticks *I. ricinus* in Europe vary from 0 to 11% for larvae, from 2 to 43% for nymphs and from 3 to 58% for adults (Hubálek and Halouzka 1998, Hulínská et al. 2007).


Since many *Borrelia* species may circulate in an area, mixed infection in ticks can be observed (Bašta et al. 1999). Ticks and their hosts may be co-infected with multiple genospecies of *B. burgdorferi* s. l. In central Europe, for example, *I. ricinus* population harbour *B. afzelii*, *B. garinii*, *B. burgdorferi* s. s. and *B. valaisiana* to varying degrees. Identification of mixed infection in ticks is possible with polymerase chain reaction (PCR).

### 1.1.3 Reservoirs

The animal reservoirs of *Borrelia burgdorferi* s. l. are numerous, mainly wild small mammals and birds. The reservoirs do not manifest signs of the disease as a result of infection. Xenodiagnosis is the classical method for determining the host infectivity of a particular vertebrate species. It is very difficult to determine the whole spectrum of reservoir hosts or the relative capacity of one or several vertebrate

In North America, Borrelia spirochetes were detected in a variety of mammalian and bird species (Anderson 1988, Anderson 1989). The white-footed mouse Peromyscus leucopus is the most competent reservoir host (Levine et al. 1985, Donahue et al. 1987, Mather et al. 1989). The while-tailed deer Odocoileus virginianus play an important role in the Lyme disease natural cycle in North America (Piesman et Gern 2004).

Small mammals

I. ricinus feeds a large variety of vertebrate hosts, but only a few dozen hosts have been currently identified as reservoirs for B. burgdorferi s. l. in Europe (Gern et al. 1998, Gern 2009). Small mammals are frequent hosts of larval and nymphal tick stages. Several species of mice, voles, rats and shrews have been shown to be competent reservoirs of B. burgdorferi s. l. in Europe (Gern et al. 1998). In particular, there is evidence that the mice Apodemus flavicollis, A. sylvaticus, A. agrarius and the vole, Clethrionomys glareolus, act as reservoirs for B. burgdorferi s. l. (Aeschlimann et al. 1986, Matuschka et al. 1992, de Boer et al. 1993, Humair et al. 1993a, 1999, Gern et al. 1994, Kurtenbach et al. 1994, 1995, 1998b, Talleklint and Jaenson 1994, Hu et al. 1997, Richter et al. 1999, Hanincová et al. 2003, Gern 2009). It was observed in Germany and France that dormice Glis glis and garden dormice Eliomys quercinus are reservoir hosts for Borrelia spp. (Matuschka et al. 1994a, 1999). Only a few studies mentioned B. burgdorferi s. l. in shrews, Sorex minutus and S. araneus (Humair et al. 1993a, Talleklint and Jaeson, 1994). Similarly, the vole Microtus agrestis (Talleklint and Jaeson, 1994), black rats Rattus rattus, and Norway rats R. norvegicus (Matuschka et al. 1994b, 1996) may also serve to infect I. ricinus ticks.

Other rodents, such as grey squirrels Sciurus carolinensis in the UK (Craine et al. 1997) and red squirrels S. vulgaris in Switzerland (Humair and Gern 1998) act as reservoirs of B. burgdorferi s. l. Several studies demonstrated that the European hedgehog Erinaceus europaeus also perpetuates B. burgdorferi s. l. in Ireland, Germany and Switzerland (Gray et al. 1994, Liebisch et al. 1996, Gern et al. 1997).

Birds

The role of birds in the maintenance of B. burgdorferi s. l. is recognized (Humair 2002). The first report in Europe of B. burgdorferi s. l. in I. ricinus ticks feeding on birds was published by Humair et al. 1993b. Olsen et al. 1993 demonstrated the existence of a transmission cycle of B. burgdorferi s. l. in seabird colonies among razorbills Alca torda. Spirochetes were reported in I. ricinus ticks collected from migratory birds in Sweden and Switzerland (Olsen et al. 1995, Poupon et al. 2006).
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Later, few studies clearly defined the reservoir role of birds: on passerine bird, blackbird *Turdus merula* (Humair et al. 1998, Taragelova et al. 2008) and on gallinaceous bird, the pheasant *Phasianus colchicus* (Kurtenbach et al. 1998a).

There are differences in the relationship between different reservoir hosts and different *Borrelia* sp. Rodents are mainly associated with *B. afzelii*, but also with *B. burgdorferi* s. s. and with *B. garinii* OspA serotype 4 whereas other *B. garinii* serotypes are associated with birds (Kurtenbach et al. 2002). *B. valaisiana* has been described only in birds and never in rodents (Humair et al. 1998, Kurtenbach et al. 1998b).

**Candidate reservoirs**

Lizards can be important hosts for *I. ricinus* larvae and nymphs and also play a role in the natural cycle of *B. lusitaniae* in Europe (Majlathová et al. 2006, Foldvari et al. 2009).

Lagomorphs play a role in the support of the enzootic cycle of *B. burgdorferi* s. l. The brown hare *Lepus europaeus* and the varying hare *L. timidus* (Jaenson and Talleklint, 1996) and European rabbit *Oryctolagus cuniculus* (Matuschka et al. 2000) contribute to the maintenance of *B. burgdorferi* s. l. in nature.

The red fox *Vulpes vulpes* is embroiled in the maintenance of *Borrelia* in nature (Liebisch et al. 1998)

Sheep was found to be a reservoir of *B. burgdorferi* s. l. in the UK, but it was found that ticks might have been infected by cofeeding (Ogden et al. 1997, Trávníček et al. 2002). Host did not necessarily become infected, but neighboring ticks serve to infect each other while feeding on the host.

**1.1.4 Domestic animals as hosts**

Besides humans, the clinical form of borreliosis occurs in domestic animals, especially dogs, horses and cattle (Burgess et al. 1987, Greene et al. 1991, Cohen et al. 1992). Most data on Lyme borreliosis in domestic animals concern dogs and horses, with isolated reports of infection in cattle, sheep and cats. Anti-borrelial antibodies have been detected in a wide range of domestic animal species. It is likely that most infections are subclinical and self-limiting. It is also possible that infections are missed or misdiagnosed because of the non-specificity of clinical symptoms.

**Lyme borreliosis in dogs**

Canine Lyme borreliosis was first reported in the USA in 1984 (Lissman et al. 1984). Typical signs are lameness combined with malaise, fatigue, listlessness,
inappetence and fever. Canine borreliosis most commonly affects the limb joints (Skotarczak et Wodecka, 2003, Skotarczak et al. 2005), with clinical manifestations such as arthritis and arthralgia (Jacobson et al. 1996). Even though the signs are the same as in humans, they are more difficult to detect in dogs and develop in relatively few of them (Levy and Magnarelli, 1992).

Diagnosis of canine borreliosis requires presence of typical clinical signs together with antibodies to \textit{B. burgdorferi} s. l. or PCR detection and evidence of exposure to ticks. Positive serology is not sufficient to diagnose borreliosis since most infection are subclinical, though serosurveys have shown that prevalence of \textit{B. burgdorferi} s. l. antibodies is usually higher in symptomatic dogs compared with healthy ones (Hovius 1999).

**Lyme borreliosis in horses**

Equine borreliosis was reported in endemic areas in the USA a few years after identification of \textit{B. burgdorferi} s. l. (Burgess et al. 1986, Burgess and Mattison 1987). Serosurveys have shown high seroprevalence in USA and in Europe (Magnarelli et al. 2000, Egenvall et al. 2001, Štefančíková et al. 2008). Clinical signs consist of malaise, fever, lameness and swollen joints (Burgess et al. 1986).

**Lyme borreliosis in cattle**

Borreliosis in cattle was reported in the USA in the 1980s (Burgess et al. 1987, Burgess, 1988). Clinical symptoms were lameness, weight loss and abortion. Lyme borreliosis in cattle is probably an infrequent disease that is difficult to diagnose due to the non-specific nature of the symptoms and there are also doubts about the specificity of the serology (Gray et al. 2002, Štefančíková et al. 2002). Cattle are not regarded as reservoir hosts of \textit{B. burgdorferi} s. l. (Gern et al. 1998) and the incidence of infected ticks on cattle pastures is probably due to the presence of other hosts in the same habitat (Gray et al. 1995).

**Lyme borreliosis in cats**

Lyme borreliosis has not been reported in cats exposed to natural infection. However, anti-\textit{Borrelia} antibodies have been detected (Magnarelli et al. 1990, May et al. 1994). Cats may be affected when they are exposed to a heavy tick challenge (Burgess 1992).
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Lyme borreliosis in sheep

*B. burgdorferi* s. l. infection of sheep rarely results in a disease; sheep have even been identified as reservoirs in the UK (see section 1.1.3.3). There are several reports of the detection of anti-borrelial antibodies in blood of sheep (Hovmark et al. 1986, Ciceroni et al. 1996, Trávníček et al. 2002). There is some evidence that adult ticks may become infected as the result of the transfer of the pathogens from infected to uninfected nymphal tick co-feeding on sheep (Ogden et al. 1997)

1.1.5 Laboratory diagnostic

Detection of infection in vertebrates can be based on host seroconversion, detection of borrelial DNA in host fluids or tissue, or recovery of live borreliae from cultured host tissue. Host seroconversion demonstrates that the vertebrate was exposed to spirochetes but does not confirm that a viable infection was established. Likewise, detection of borrellial DNA in host tissue or fluids, although allowing detection of the genospecies involved, is not necessarily indicative of a viable infection. It is very useful to have standardized and dependable tests to ascertain borrelial infection, with ability to distinguish between active and inactive infection (Gray et al. 2002, Piesman and Gern 2004).

Methods for detection of *B. burgdorferi* s. l. are direct and indirect. Direct methods detect the bacteria or its parts. The most often used ones are cultivation, microscopy and molecular methods. Indirect methods mainly serological are based on detecting bacteria antibodies.

Indirect laboratory methods detect only certain parts of borrelial cell such as the outer surface and other proteins. Examples are immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and western blot test (WB) (Piesman and Gern 2004). Official recommendation for human serology (from Centers for Disease Control) is to use a two-step procedure for detection of Lyme disease – first, to perform a sensitive screening test, such as ELISA and, if the result is positive or equivocal, a WB test to confirm the results (Shapiro 2008).

IFA may indicate living borreliae by showing the typical helical morphology of the spirochaete. It is also possible to combine culture and IFA to obtain clear evidence of living borreliae.

* Borrelia infection should be confirmed by isolation of *B. burgdorferi* s. l. from the tissue and body fluids involved. Although *B. burgdorferi* s. l. grows relatively well under laboratory conditions, spirochetes are not easily recovered from clinical specimens other than skin biopsy samples. Culture is rather costly and time consuming to be used for routine detection of borreliae. However, it provides a large number of cells that facilitates subsequent identification and characterization e.g. by a polymerase chain reaction (PCR) (Gray et al. 2002).
Detection of borrelial genetic material by PCR has been acclaimed for its high sensitivity and specificity. However, the value of detection of borreliae by this method in tissues and fluids from vertebrates has not yet been unequivocally established. They demonstrate infection but not the presence of living agents, although the detection of RNA by PCR may indicate a living organism since RNA degrades very rapidly (Dumler 2003).

1.2 Anaplasmosis

The second tick-borne disease addressed in this work is Anaplasmosis. Compared to borreliosis, there are less published reports on Anaplasma because it was discovered much later. Human granulocytic anaplasmosis (HGA) was first identified in 1990 in USA, in patient who died with a severe febrile illness two weeks after a tick bite (Chen et al. 1994). In Europe, antibodies against Anaplasma phagocytophilum were described in 1995 in the Swiss population (Brouqui et al. 1995). First documented HGA case in Europe was reported by Petrovec et al. 1997 in Slovenia. Anaplasmosis in domestic ruminants is also called tick-borne fever and has been mentioned in 1932 (Gordon et al. 1932). HGA agent is maintained in nature in a tick-ruminant-rodent cycle. Humans are involved only as accidental “dead-end” hosts (Blanco and Oteo 2002).

The agent A. phagocytophilum, may cause infection in several animal species including human. Anaplasmosis may cause high fever, inappetence, malaise, cytoplasmatic inclusions in granulocytic neutrophils, neutropenia and thrombocytopenia (Rikihisa 1991, Greig et al. 1996, Egenvall et al. 1997, Engvall and Egenvall 2002). Anaplasmosis is seldom fatal unless there are complications by other infection. In humans, clinical manifestations range from a mild self-limited flu-like illness to a life-threatening infection. Most human infection probably results in minimal or no clinical manifestation (Dumler et al. 2005).

1.2.1 Anaplasma phagocytophilum

A. phagocytophilum was described in 1994 in the USA (Bakken et al. 1994, Chen et al. 1994). The agent was recognized by molecular amplification and DNA sequencing and was initially named Human granulocytic ehrlichiosis (HGE) agent. Recently, the families Rickettsiaceae and Anaplasmataceae were complete revised and these bacteria: Ehrlichia phagocytophilum, E. equi and the HGE agent were reclassified as a single species, Anaplasma phagocytophilum (Dumler et al. 2001).

A. phagocytophilum has been detected in ticks and mammals in many European countries (Strle 2004). Seroprevalence rates in European countries range
from zero to up to 28% of the examined human population (Strele 2004).

*Anaplasma phagocytophilum* is small (0.2 – 1.0 µm) obligate intracellular bacteria with a gram-negative cell wall (Walker and Dumler 1996, Parola and Raoult 2001). This bacterium resides in an early endosome in granulocytic neutrophils, where *Anaplasma* obtains nutrients for fission and grows into a cluster called a morulae (Lin and Rikihisa 2003).

### 1.2.2 Vectors

The main vector of *A. phagocytophilum* in Europe is *Ixodes ricinus* (Parola and Raoult 2001, Strele, 2004). The prevalence of *A. phagocytophilum* infection in *I. ricinus* in Europe varies from area to area and between development stages of the tick (Lillini et al. 2006). Occurrence in nymphs has been found to vary between 0.25-25% (Walker et al. 2001). Prevalence is usually higher in adult ticks than in nymphs and ranges from zero to 30% (Pusterla et al. 1999a, Liz et al. 2000, Strele 2004). Tick is infected after feeding on an infected host. The bacterium is passed transstadially but not transovarially (Dumler et al. 2001).

*A. phagocytophilum* has been associated with other ticks such as *Haemaphysalis punctata* (Barandika et al. 2008), *I. persulcatus* (Alekseev et al. 1998), *I. trianguliceps* (Ogden et al. 1998a) and *Rhipicephalus sanguineus* (Alberti et al. 2005).

In the USA, *A. phagocytophilum* has been often associated with *I. scapularis* and with *I. pacificus*, and these may serve as the primary vectors (Barlough et al. 1997a, Chang et al. 1998).

### 1.2.3 Reservoirs

There is field evidence that sheep are natural hosts for *A. phagocytophilum* in the UK (Ogden et al. 1998a,b, Ogden et al. 2002). In the USA rodents, particularly white-footed mice *P. leucopus* (Bunnell et al. 1998), and white-tailed deer *O. virginianus* (Belongia et al. 1997) are involved as natural reservoirs for *A. phagocytophilum*.

**Small mammals**

Wild rodents in Europe have been suggested to be competent reservoirs for *A. phagocytophilum* (Ogden et al. 1998a, Liz et al. 2000). Long-tailed mice *A. sylvaticus*, yellow-necked mice *A. flavicollis*, common shrew *S. araneus*, and bank voles *C. glareolus* were found to be likely natural reservoir for *A. phagocytophilum* (Liz et al. 1998, Liz 2002).
Candidate reservoirs


A study from Slovenia revealed by PCR that red deer *Cervus elaphus* and roe deer *Capreolus capreolus* are infected with *A. phagocytophilum* in about 86% of cases, and the prevalence of IFA antibodies was found to be 35% and 94%, respectively (Petrovec et al. 2002). Red foxes *V. vulpes* and wild boar *Sus scrofa* were also found to be PCR positive (Petrovec et al. 2003). Infection by *Anaplasma* has been also identified in European bison (Grzeszcuk et al. 2003), donkey (de la Fuente et al. 2005b), and moose (Jenkins et al. 2001). Antibodies have been detected in hare (Groen et al. 2002) and Eurasian lynx (Ryser-Degiorgis et al. 2005).

### 1.2.4 Domestic animals as hosts

*A. phagocytophilum* is known to cause granulocytic anaplasmosis in humans (Petrovec et al. 1997) and domestic animals such as horses (Bjoersdorff 1990, Engvall et al. 1996, Engvall and Egenvall 2002), dogs (Bellstrom 1989, Engvall et al. 1996, Engvall and Egenvall 2002, Skotarczak 2003, Lester et al. 2005, Poitout et al. 2005), cats (Bjoersdorff et al. 1999), cattle (Engvall et al. 1996), and llamas (Barlough et al. 1997b). *A. phagocytophilum* has been found to persist in sheep (Stuen et al. 1998).


**Anaplasmosis in dogs**

*A. phagocytophilum* often causes chronic disease in dogs with non-specific clinical findings. Clinical and haematological findings in dogs are fever, inappetence, joint swelling and pain, lameness, stiffness, neurologic inflammation, thrombocytopenia and neutropenia. Canine anaplasmosis was reported from many European countries (Sweden, Greece, Italy, Slovenia, Austria, Poland, Switzerland, and Czech Republic) (Lillini et al. 2006).
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Anaplasmosis in horses

*A. phagocytophilum* in horses was reported in many European countries (Great Britain, Denmark, Sweden, Switzerland, France, Germany, Czech Republic and Italy). It was detected by serological and molecular methods, and also by positive findings in buffy coat smears. Hematological findings on *Anaplasma*-positive horses showed thrombocytopenia and leukocytosis (Bjoersdorff 1990, Bjoersdorff et al. 2002, Lillini et al. 2006, Zeman and Jahn 2009).

Anaplasmosis in ruminants

In domestic ruminants *A. phagocytophilum* causes a disease known as a tick-borne fever (TBF). *A. phagocytophilum* in sheep causes very high fever, reduced milk yield and abortion in pregnant animals. Laboratory findings are neutropenia and cytoplasmatic inclusion with more than 95% of neutrophils infected (Garcia-Perez et al. 2003).

1.2.5 Laboratory diagnostic

The majority of *Anaplasma* infection is identified by indirect laboratory methods. The most frequent used method is the IFA test. While this test is often used, reactivity in samples with anticytoplasmic antibodies or with other autoimmune antibodies may confound interpretation, and the correlation of results from different laboratories and different commercial tests may be difficult (Blanco and Oteo 2002).

*A. phagocytophilum* is visible as a cluster of small cocci in cytoplasm of neutrophils in the peripheral blood on a Wright- or Giemsa-stained smear. The characteristic cytoplasmatic inclusion in neutrophils can be detected in between 25 and 80% of patients during the active stage of HGA (Dumler and Brouqui 2004).

This bacterium is an obligate intracellular parasite that can only be cultivated in cell lines derived from bone marrow myeloid progenitors, for example the human HL-60 promyelocytic leukemia cell line (Goodman et al. 1996). Cultivation may require several days to several weeks. Morphologic identification in culture still requires confirmation for example by PCR (Dumler and Brouqui 2004).

Detection of *Anaplasma* genetic material by PCR demonstrates the presence of *A. phagocytophilum* nucleic acid in peripheral blood but not the presence of living agents. HGA is one of the rare infectious diseases that was first confirmed based upon molecular tests rather than culture or serology (Chen et al. 1994). The first tests used were based on an amplification of the highly conserved 16S rRNA gene, followed by a second stage that utilized primers suspected to anneal only to *A. phagocytophilum rrs*-specific sequence (Dumler et Brouqui 2004).
1.3 Aims of this thesis

- Determine the prevalence of *B. burgdorferi* s. l. and *A. phagocytophilum* in vectors, confirmed and candidate reservoirs, and hosts in the Czech Republic. Compare the results with other European studies.

- Compare the prevalence of *B. burgdorferi* s. l. and *A. phagocytophilum* in different rodent species, detected by molecular and serological methods.

- Determine *Borrelia* genospecies occurring in their important reservoir hosts, rodents, using different molecular methods.

- Study the prevalence of *B. burgdorferi* s. l. and *A. phagocytophilum* in domestic animals, confirm whether there is a difference between symptomatic and asymptomatic hosts. Study and describe borreliosis symptoms.

- Compare *A. phagocytophilum* variants occurring in wild and domestic animals and ticks by direct sequence analysis.

- Determine, implement and test suitable diagnostic methods for detection of borreliosis and anaplasmosis from blood and tissue samples.

- Implement molecular detection methods for *B. burgdorferi* s. l. and *A. phagocytophilum* using real-time PCR and evaluate its advantages and disadvantages with respect to other molecular methods.
Chapter 2

Overview and results

The main part of this thesis consists of four papers which have been published in peer-reviewed international journals and that we are including verbatim. We give here a brief summary of each paper and its relevance.

2.1 Molecular and serological evidence of Borrelia burgdorferi sensu lato in wild rodents in the Czech Republic.

The aim of the first paper (Kybicová et al. 2008) was to determine the frequency and spatial distribution of the Borrelia species in wild rodents in the Czech Republic. In total, 293 muscle tissue samples and 106 sera from 293 wild rodents captured in North Bohemia and North-East and South Moravia were examined for the presence of Borrelia spp. and antibodies. Apart from a small sample preliminary study (Hulínská et al. 2002), the prevalence has not yet been determined using PCR methods and only serological data have been available (Vostál and Žákovská 2003).

Infection with B. burgdorferi s. l. was found in 16.4% of the muscle samples. The most abundant genospecies was B. afzelii (11.3%), followed by B. burgdorferi s. s. (4.8%) and B. garinii (0.7%). Borrelia infection was more frequently observed in C. glareolus than in Apodemus spp. Sera were analyzed using an ELISA test, yielding the total seropositivity rates of 24.5% for anti-Borrelia IgM antibodies and 25.5% for IgG antibodies. Total seroprevalence was higher in Apodemus spp. than in C. glareolus. Our data indicate that in the Czech Republic small wild rodents can serve as hosts for B. burgdorferi s. s. as well as for B. afzelii.
2.2 Detection of Anaplasma phagocytophilum and Borrelia burgdorferi sensu lato in dogs in the Czech Republic.

The second paper (Kybicová et al. 2009) presents molecular, serological and clinical findings for dogs that were naturally infected with A. phagocytophilum or B. burgdorferi s. l. in the Czech Republic. In total, blood samples from 296 dogs and 118 engorged ticks were examined. Dogs are important domestic hosts of A. phagocytophilum and B. burgdorferi s. l. Data on vector-borne infections in dogs can provide important information for the potential of human infection in a particular geographic location (Duncan et al. 2005). Data on the prevalence of infection with A. phagocytophilum and B. burgdorferi s. l. in dogs in the Czech Republic was missing, except for a serology study by Pejchalová et al. 2006 and a few case reports.

Ten (3.4%) dogs were PCR-positive for A. phagocytophilum. Morulae of A. phagocytophilum in granulocytes were found in two of these dogs. Nine of the PCR-positive dogs had clinical signs related to anaplasmosis. Statistically significant differences in the PCR detection rates were found between breeds and between symptomatic and asymptomatic dogs. Infection with B. garinii was detected by PCR in a dog with meningoencephalitis. DNA of A. phagocytophilum and B. burgdorferi s. l. (B. garinii or B. afzelii) was detected in 8.5% and 6.8% of ticks, respectively. IgG seropositivity to A. phagocytophilum was 26%. Significant differences were found with respect to breed and gender. IgM and IgG antibodies to B. burgdorferi s. l. were detected in 2.4% and 10.3% of dogs, respectively. Our findings suggest that the exposure to B. burgdorferi s. l. exists in dogs in the Czech Republic and exposure to A. phagocytophilum is common.

2.3 Clinical and Diagnostic Features in Three Dogs Naturally Infected with Borrelia spp.

The third paper (Schánilec et al. 2010) presents clinical and neurological signs, laboratory abnormalities, serologic and/or molecular findings in three dogs from the region of Brno in the Czech Republic. All dogs were naturally infected with Borrelia burgdorferi sensu lato. The evidence of borrelial infection was proved by serial blood sampling for IgM and IgG anti-borrelial antibodies and plasma PCR.

All three dogs showed neurological signs (two of them had meningoencephalomyelitis, one had a seizure connected with a progressive renal disease). Their history, clinical signs, diagnostic procedures and treatment are described.
Two of the infected dogs died and only one with meningoencephalomyelitis survived. This paper shows that borrelial infection must be considered, not only in cases with febrile and orthopaedic signs but also in many other clinical syndromes.

2.4 Detection of *Anaplasma phagocytophilum* in animals by real-time polymerase chain reaction.

The aim of the fourth paper (Hulínská et al. 2004) is the detection of *A. phagocytophilum* in wild and domesticated animals and the identification of phylogenetic relationships of different variants of this bacterium. Six published conventional methods targeting 16S rDNA fragments were adapted for a real-time polymerase chain reaction, using the LightCycler real-time PCR technique.

Initial screening of samples from 419 animals found 37 *Anaplasma* positives, later confirmed with several different primers and a TaqMan probe. The nucleic acid of *Anaplasma* sp. was detected in a higher percentage of cases in members of the deer family, hares, bank voles and mice (12.5–15%) than in foxes, boars, cows, and horses (around 4–6%).

At the time of writing there were only a small number of studies on animal reservoirs for *A. phagocytophilum* in Europe in the literature. To the best of our knowledge, the direct PCR sequence analysis from samples of a large number of wild animals was reported here for the first time. We succeeded in properly selecting primers for direct sequencing that differentiate well between the different variants of *A. phagocytophilum*. To analyze the relationships between these variants, we sequenced the PCR products from our samples and formed a phylogenetic tree using as a reference the sequence of *Ehrlichia equi*. Mutual identity of the sequencing ranged from 99% to 100%.

2.5 Summary of the results

Prevalence of *B. burgdorferi* s. l. and *A. phagocytophilum* detected by molecular (PCR) and serological (ELISA and IFA) methods in vectors, reservoirs, candidate reservoirs and domestic hosts is shown in Table 2.1. For more details please see the articles mentioned in the previous section.
Table 2.1: Summary of detected prevalences. B. b. and A. p. stand for *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophylum*, respectively.

<table>
<thead>
<tr>
<th></th>
<th>PCR</th>
<th>Serology</th>
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<tbody>
<tr>
<td>Vectors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ticks from rodents</td>
<td>9.8%</td>
<td></td>
</tr>
<tr>
<td>ticks from dogs</td>
<td>6.8%</td>
<td>8.5%</td>
</tr>
<tr>
<td>Reservoirs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bank voles</td>
<td>25.3%</td>
<td>13.3%</td>
</tr>
<tr>
<td>mice</td>
<td>8.1%</td>
<td>15.0%</td>
</tr>
<tr>
<td>Candidate reservoirs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>deer family</td>
<td>12.9%</td>
<td></td>
</tr>
<tr>
<td>hares</td>
<td>12.5%</td>
<td></td>
</tr>
<tr>
<td>foxes</td>
<td>4.0%</td>
<td></td>
</tr>
<tr>
<td>boars</td>
<td>4.4%</td>
<td></td>
</tr>
<tr>
<td>Domestic hosts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cows</td>
<td>5.5%</td>
<td></td>
</tr>
<tr>
<td>horses</td>
<td>5.0%</td>
<td></td>
</tr>
<tr>
<td>dogs</td>
<td>0.3%</td>
<td>3.4%</td>
</tr>
<tr>
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</tr>
</tbody>
</table>

— 16 —
Chapter 3

Molecular and serological evidence of *Borrelia burgdorferi* sensu lato in wild rodents in the Czech Republic.

Molecular and Serological Evidence of *Borrelia burgdorferi* sensu lato in Wild Rodents in the Czech Republic

K. KYBICOVÁ, Z. KURZOVÁ, and D. HULÍNSKÁ

**ABSTRACT**

The aim of the present study was to determine the frequency and spatial distribution of the *Borrelia* species in wild rodents in the Czech Republic. In total, 293 muscle tissue samples and 106 sera from 293 wild rodents captured in North Bohemia and North-East and South Moravia were examined for the presence of *Borrelia* spp. and antibodies. Muscle samples were investigated with real-time polymerase chain reaction (PCR) with a recA primer set, with DNA quantification and melting curve analysis, and with restriction fragment length polymorphism (RFLP) analysis of the 5S–23S rDNA intergenic spacer. Infection with *Borrelia burgdorferi* sensu lato was found in 16.4% of the muscle samples. The most abundant genospecies was *Borrelia afzelii* (11.3%), followed by *Borrelia burgdorferi* sensu stricto (4.8%) and *Borrelia garinii* (0.7%). *Borrelia* infection was more frequently observed in *Clethrionomys glareolus* than in *Apodemus* spp. Sera were analyzed with an enzyme-linked immunosorbent assay (ELISA) test, yielding the total seropositivity rates of 24.5% for anti-*Borrelia* IgM antibodies and 25.5% for IgG antibodies. Total seroprevalence was higher in *Apodemus* spp. than in *C. glareolus*. In conclusion, our data indicate that in the Czech Republic small wild rodents can serve as hosts for *B. burgdorferi* s. s. as well as for *B. afzelii*.

Key Word: Borrelia

**INTRODUCTION**

*Lyme borreliosis is a multi-organ disease of mammals in the northern hemisphere. It is caused by Borrelia burgdorferi sensu lato (s. l.), which currently includes 11 genospecies, three of which are known to be pathogenic: Borrelia burgdorferi sensu stricto (s. s.), Borrelia afzelii, Borrelia garinii (Johnson et al. 1984; Baranton et al. 1992; Canica et al. 1993). Occasionally, Borrelia valaisiana, Borrelia lusitaniae, Borrelia bisetosa, and Borrelia spielmanii were detected in patients (Picken et al. 1996; Ripkiema et al. 1997; Wang et al. 1999; Collares-Pereira et al. 2004). Nevertheless, the association of these and other Borrelia species with Lyme disease has not yet been confirmed.*

Studies of the ecology of Lyme borreliosis have demonstrated that the persistence of *Borrelia* in endemic areas depends on the presence of reservoir hosts (Gern et al. 1998; Humair et al. 1998). In North America, *Borrelia* spirochetes have been detected in a variety of mammalian and bird species (Anderson 1988, 1989). The white-footed mouse *Peromyscus leucopus* is the most competent reservoir host (Levine et al. 1985; Donahue et al. 1987; Mather et al. 1989). In Europe, wild rodents were studied in various enzootic areas (de Boer et al. 1993; Humair et al. 1993; Tallekint and Jaensson 1994; Kurtenbach et al. 1995), and several rodent species have been suggested as natural reservoirs for *B. burgdorferi* s. l. (Matuschka et al. 1992, 1997), especially the woodmouse *Apodemus sylvaticus*, the yellow-necked mouse *Apodemus flavicollis* (Aeschlimann et al. 1986), and the bank vole *Clethrionomys glareolus* (Hovmark et al. 1988).
Molecular identification methods have made it possible to determine specific associations between hosts and *Borrelia* genospecies. In small rodents, *B. afzelii* dominated in Switzerland (Humair et al. 1995; Hu et al. 1997), whereas in the United Kingdom, *B. burgdorferi* s. s. prevailed (Kurtenbach et al. 1998b). Generally, the most prevalent genospecies in rodents in Europe appears to be *B. afzelii*, followed by *B. burgdorferi* s. s., while *B. garinii* is rare. *B. afzelii* seems to be preferentially transmitted by rodents (Hu et al. 1997; Humair et al. 1995, 1998, 1999). Most strains of *B. garinii* and *B. valaisiana* display resistance to the bactericidal activity of avian complement and are therefore considered bird-associated *Borrelia* ecotypes (Kurtenbach et al. 1998a; Hanincova et al. 2003). However, some subtypes of *B. garinii* can also infect European rodents (Richter et al. 1999; Gray et al. 2000; Huegli et al. 2002).

The aim of the present study was to assess the prevalence of *B. burgdorferi* s. l. infections in wild rodents in the Czech Republic, and to determine the genospecies within *B. burgdorferi* s. l. associated with these hosts. Apart from a small sample preliminary study (Hulinska et al. 2002), the prevalence has not yet been determined using polymerase chain reaction (PCR) methods, and only serological data have been available (Vostal and Zakovska 2003).

**MATERIALS AND METHODS**

**Locations studied**

Rodents were collected in May and July 2003 and between October and December 2004 in wooded areas (mixed forest) and nearby fields in three different areas of the Czech Republic: area 1, the terrain surrounding Děčín, a town in North Bohemia (50°43'N, 14°7'E; altitude 300–500 m); area 2, the Vsetín highlands in North-East Moravia (49°22'N, 18°14'E; altitude 500–600 m); and area 3, the terrain surrounding Brno, a large city in South Moravia (49°3'N, 16°38'E; altitude 100–300 m) (Fig. 1). The average temperature and average rainfall were 16.8°C and 70 mm in May 2003, 20°C and 66 mm in July 2003, 10.2°C and 73 mm in October 2004, 3.6°C and 95 mm in November 2004, and −0.1°C and 19 mm in December 2004.

**Rodent capture**

Rodents were captured alive with iron box traps or dead with collapsible traps. The traps were spaced 5 m apart, baited with hay, grains, and pieces of vegetables, and exposed overnight. In laboratory each animal was classified into species, weighed, measured and then euthanized. Samples from muscle tissue (from all study areas) and blood sera (from ar-

FIG. 1. Geographical representation of three localities in the Czech Republic from which rodent samples were obtained.
ea 1 and 2) were extracted and stored in 1.5 ml microcentrifuge tubes at ~20°C prior to DNA extraction and the enzyme-linked immunosorbent assay (ELISA) test.

DNA extraction

Total DNA was isolated with the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Approximately 20 mm³ of tissue was transferred to a tube containing 180 µL of lysis buffer and 20 µL of proteinase K and lysed at 56°C until the tissue was completely lysed (2–3 h). DNA was extracted according to the manufacturer’s instructions and stored at ~20°C until PCR amplification was performed.

PCR and real-time PCR amplification

All samples were screened by standard PCR with the LD primer set targeting the 16S rDNA gene (Marconi et al.1992). The PCR amplification was performed in a Peltier cycler (MJ Research, MA, USA). The reaction mixture consisted of 5 µL of DNA extract as a template and 1 µM solution of each primer (Generi Biotech, Czech Republic) in a total volume of 25 µL Hot Start Master mix (Qiagen, Hilden, Germany). Cycling conditions involved an initial 15 minute denaturation at 95°C, followed by 37 cycles, each consisting of a 1 minute denaturation at 94°C, a 30 second annealing at 52°C, and a 1.5 minute extension at 72°C. These 37 cycles were followed by a 7 minute extension at 72°C. The PCR products were separated by electrophoresis in 1% agarose gel and stained with ethidium bromide and visualized by UV transilluminator. LD primers produce a 222-bp sequence from the recA gene with the following cycling conditions: an initial denaturation of 5 minutes at 95°C, 40 cycles consisting of a 5 second denaturation at 95°C, 5 second annealing at 60°C, and a 11 second extension at 72°C. After the final PCR cycle, the PCR products were denatured at 95°C, annealed at 60°C, and then slowly heated to 95°C.

We performed DNA relative quantification for the recA primers. The standard curve and the amplification plot were derived from a ten-fold dilution series of the positive control B. afzelii strain Kc90. The concentration of Borrelia DNA in the positive control was determined spectrophotometrically.

RFLP analysis

For further characterization, positive DNA samples were tested by RFLP analysis with 5S (rrfA)-23S (rrlB) rDNA intergenic spacer primers (222 ~ 255 bp) (Derdáková et al. 2003) under the following cycling conditions: an initial denaturation of 15 minutes at 95°C, five cycles of 94°C for 15 seconds, 61°C (for the first cycle with the temperature decreasing by 0.2°C/cycle) for 25 seconds, and 72°C for 30 seconds, followed by five cycles of 94°C for 15 seconds, 60°C (for the first cycle with the temperature decreasing by 0.4°C/cycle) for 25 seconds, and then 30 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, followed by a 10 minute extension at 72°C. The reaction mixture consisted of 2.5 µL of DNA extract as a template and a 1 µM solution of each primer (Generi Biotech, Czech Republic) in a total volume of 25 µL of Hot Start Master Mix (Qiagen, Hilden, Germany). DNAs of B. afzelii strain Kc90, B. garinii strain M192, B. burgdorferi s. s. strain B31, and B. valaisiana strain E117 were used as positive controls. For each positive sample, 10–20 µL of amplified DNA was digested at 65°C for 2 h in a solution containing 5 U of Tru 9l (10,000 U/mL) and 1× buffer M (Roche Molecular Biochemicals, Mannheim, Germany), containing Taq DNA polymerase, SYBR-Green I, deoxynucleoside triphosphate mix, and 3 mM MgCl₂. The samples were tested with primers nTM17F and nTM17R (Morrison et al. 1999) to amplify a 222-bp sequence from the recA gene with the following cycling conditions: an initial denaturation of 5 minutes at 95°C, 40 cycles consisting of a 5 second denaturation at 95°C, 5 second annealing at 60°C, and a 11 second extension at 72°C. After the final PCR cycle, the PCR products were denatured at 95°C, annealed at 60°C, and then slowly heated to 95°C.

We performed DNA relative quantification for the recA primers. The standard curve and the amplification plot were derived from a ten-fold dilution series of the positive control B. afzelii strain Kc90. The concentration of Borrelia DNA in the positive control was determined spectrophotometrically.
ELISA

The sera were examined by a modified ELISA as described elsewhere (Stefancikova et al. 2004). The whole cell lysate of the B. afzelii strain Kc90 was used as an antigen for detection of anti-Borrelia antibodies. Mouse sera with absorbance values of less than 0.4 were used as negative controls. Sera from naturally infected rodents, which were positive in repeated titrations, served as positive controls. All these rodents were also PCR positive. The cut-off was determined as 3 standard deviations above the mean optical density of the negative controls (Magnarelli et al. 1988; Stefancikova et al. 2004).

Statistical analysis

Statistical significance of differences (at a level of \(p < 0.05\)) in the prevalence among areas, rodent species, and Borrelia genospecies was analyzed by analysis of variance (ANOVA) with a repeated measures post-hoc test.

RESULTS

Wild rodents

A total of 293 wild rodents were trapped; they were from the following species: Apodemus flavicollis (yellow-necked mouse, 105 individuals), Apodemus sylvaticus (wood mouse, 42), Clethrionomys glareolus (bank vole, 95), Sorex araneus (common shrew, 10), Pitymys subterraneus (earth vole, 6), Microtus arvalis (common vole, 33) and Microtus agrestis (field vole, 2). In total, 293 muscle tissue samples and 106 sera samples (from areas 1 and 2) were tested.

Detection of B. burgdorferi s. l. in rodents by areas and species

Tissue samples were screened for B. burgdorferi s. l. by PCR. The rate of infection with Borrelia burgdorferi s. l. was 16.4% (48 of 293 samples were positive). The positive samples were analyzed by real-time PCR and PCR-RFLP genotyping. Three genospecies were detected: B. afzelii, B. burgdorferi s. s., and B. garinii. B. afzelii was the most abundant (11.3%), followed by B. burgdorferi s. s. (4.8%); B. garinii was rare (0.7%) (Table 1).

We found the highest percentage of infected rodents (17.1%; 15 of 82 animals) in area 1, where three genospecies were present: B. afzelii (12 animals), B. garinii (2 animals), and one mixed infection with B. afzelii and B. burgdorferi s. s. In area 2, the positivity rate was 13.8% (11/80), and all infections were caused by B. afzelii. In area 3, Borrelia DNA was detected in 16.8% of animals (22/131), with 9 rodents infected by B. afzelii and 13 infected by B. burgdorferi s. s. The differences between areas were not statistically significant.

The positivity rate was significantly higher (\(p < 0.05\)) in C. glareolus (25.3%; 24/95) than in A. flavicollis (7.6%; 8/105), and A. sylvaticus (9.5%; 4/42). The rates of other infected rodents were as follows: M. arvalis, 21.2% (7/33); P. subterraneus, 50% (3/6); M. agrestis, 100% (2/2),

<table>
<thead>
<tr>
<th>Rodent</th>
<th>No. of examined animals</th>
<th>No. (%) of positive</th>
<th>Number of rodents infected with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B.a.</td>
</tr>
<tr>
<td>Clethrionomys glareolus</td>
<td>95</td>
<td>24 (25.3)</td>
<td>17</td>
</tr>
<tr>
<td>Apodemus flavicollis</td>
<td>105</td>
<td>8 (7.6)</td>
<td>1</td>
</tr>
<tr>
<td>Apodemus sylvaticus</td>
<td>42</td>
<td>4 (9.5)</td>
<td>3</td>
</tr>
<tr>
<td>Sorex araneus</td>
<td>10</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Pitymys subterraneus</td>
<td>6</td>
<td>3 (50)</td>
<td>3</td>
</tr>
<tr>
<td>Microtus arvalis</td>
<td>33</td>
<td>7 (21.2)</td>
<td>6</td>
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<tr>
<td>Microtus agrestis</td>
<td>2</td>
<td>2 (100)</td>
<td>2</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area 1</td>
<td>82</td>
<td>15 (18.3)</td>
<td>12</td>
</tr>
<tr>
<td>Area 2</td>
<td>80</td>
<td>11 (13.8)</td>
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<tr>
<td>Area 3</td>
<td>131</td>
<td>22 (16.8)</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>293</td>
<td>48 (16.4)</td>
<td>32</td>
</tr>
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</table>
S. araneus, and 0% (0/10). All three *Borrelia* genospecies were detected in *C. glareolus*: *B. afzelii* in 17 animals, *B. burgdorferi s.s.* in 5 animals, and *B. garinii* in 2 animals. In *A. flavicollis*, *B. afzelii* and *B. burgdorferi s.s.* were detected in 3 and 5 animals, respectively. *A. sylvaticus* was positive once for *B. afzelii* and twice for *B. burgdorferi s.s.* In all other infected rodent species, only *B. afzelii* was detected, except for *M. arvalis*, with one animal infected by *B. burgdorferi s.s.* (Table 1).

Detection of *B. burgdorferi s.l.* in rodents by RT-PCR and RFLP

DNAs from *Borrelia* controls and 48 positive samples were subjected to the recA gene LightCycler PCR and melting curve analysis. With uTM17F and nTM17R used as primers, mean melting temperatures for controls strains of *B. afzelii*, *B. burgdorferi s.s.*, and *B. garinii* were 83.5°C, 84.6°C, and 80.8°C, respectively. All unspecific products melted at temperatures below 80°C. The estimated concentration of *B. burgdorferi s.l.* DNA in rodent samples ranged between 4.2 and 42 pg/µL. This corresponds to 20 ~ 200 spirochetes per nanogram of the original tissue, using the average nucleotide MW 330 pg/pmol and the *Borrelia* DNA length of 1.5 Mb (Lederer et al. 2005).

The RFLP restriction pattern of *B. afzelii* is ~105 bp, ~70 bp, 68 bp, and 20 bp; that of *B. burgdorferi s.s.* is ~105 bp, ~70 bp, 38 bp, and 29 bp; and that of *B. garinii* is ~105 bp, 97 bp, and ~80 bp (Fig. 2). The RFLP study confirmed the findings of real-time PCR and could distinguish between *B. afzelii* and *B. burgdorferi s.s.* We also confirmed the mixed infection with *B. afzelii* and *B. burgdorferi s.s.*

Serological findings

We analyzed 106 rodent sera: 34 from *A. flavicollis*, 11 from *A. sylvaticus*, 56 from *C. glareolus*, 2 from *P. subterraneus*, and 3 from *M. agrestis*. The total seropositivity rates were 24.5% (26/106) for anti-*Borrelia* IgM antibodies and 25.5% (27/106) for anti-*Borrelia* IgG antibodies. As for the rodent species, the highest prevalence of IgM and IgG antibodies was observed in *Apodemus* spp. (26.7% and 31.1%, respectively), followed by *C. glareolus* (16.1% and 21.4%, respectively) (Table 2). All animals of the *P. subterraneus* and *M. agrestis* species were IgM positive, with *P. subterraneus* showing IgG positivity only once (1/3). Seroprevalence in other rodent species was not determined because of the small number of samples. In area 1 there were 14 IgM, and 15 IgG positive sam-

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**FIG. 2.** RFLP profiles for the SS-23S rDNA intergenic spacer of samples for rodents and positive controls. Lanes 11, 12, and 13 in both panels contain positive controls for *B. afzelii*, *B. garinii*, and *B. burgdorferi s.s.*, respectively. In the left-hand panel in lanes 1, 2, 5, 6–10 there are positive samples from rodents corresponding to *B. afzelii*, in lane 3 there is a positive sample from a rodent with *B. garinii*, and lane 4 contains a co-infection with *B. afzelii* and *B. burgdorferi s.s.* In the right-hand panel in lanes 14–25 there are positive samples corresponding to *B. burgdorferi s.s.* Lane M1 contains a PCR 20Bp Low Ladder Marker (Sigma) and lane M2 contains a Wide Range DNA Marker (Sigma).
DISCUSSION

Many species have been reported to serve as competent reservoirs of B. burgdorferi s. l. in Europe, in particular rodents of the genera Clethrionomys and Apodemus (Matuschka et al. 1992; Hu et al. 1997; Humair et al. 1999; Hanincova et al. 2003). In the present study, we found Borrelia infection to be more common in C. glareolus than in Apodemus spp. (Zore et al. 1999). The most frequently captured rodent species were A. flavicollis and C. glareolus, the most abundant sylvan rodents in the Czech Republic and its neighboring states (Hanincova et al. 2003).

B. afzelii, B. burgdorferi s. s., and B. garinii were detected in the present study. To our best knowledge, this is the first report of the presence of B. burgdorferi s. s. in rodents in the Czech Republic. In our data, the majority of infections were caused by B. afzelii (Humair et al. 1995, 1998, 1999; Hu et al. 1997; Hanincova et al. 2003). A possible explanation is a reduced and shorter-lived infectivity of B. burgdorferi s. s. in comparison with B. afzelii, which is better adapted to rodent hosts (Richter et al. 2004). However, B. burgdorferi s. s. was detected in small rodents in the United Kingdom, Poland, Ireland, and Switzerland (Kurtenbach et al. 1998b; Gray et al. 1999; Humair et al. 1999; Michalik et al. 2005). B. burgdorferi s. s. seems to be less specialized and may be maintained by both avian and rodent hosts (Kurtenbach et al. 1998a). We have demonstrated one case of coinfection with B. afzelii and B. burgdorferi s. s. in A. sylvaticus (Zore et al. 1999).

The presence of Borrelia was previously studied in ear biopsies and internal organs of small mammals (spleen, heart, liver, and urinary bladder) (Humair et al. 1993; Zore et al. 1999; Hanincova et al. 2003; Christova et al. 2005). As ear biopsies may not reveal the full diversity of the infection, (Richter et al. 1999; Hanincova et al. 2003), we have used muscle samples, observing an infection rate of 16.4% (Kurtenbach 1998b; Christova et al. 2005).

Real-time PCR analysis of the recA gene (Fraser et al. 1997) is a rapid method for detection of B. burgdorferi s. l., with sensitivity similar to that of nested PCR (Pietila et al. 2000; Wang et al. 2003). In the present study, the concordance between real-time PCR and RFLP analysis was 97.6%. The melting curve analysis permits differentiation of B. garinii from B. afzelii and B. burgdorferi s. s., but it does not make it possible to distinguish between B. afzelii and B. burgdorferi s. s., as the melting temperature difference can be as small as 1°C (Mommert et al. 2001; Wang et al. 2003; Casati et al. 2004). The RFLP method not only can confirm the real-time PCR results but also is able to distinguish between B. afzelii and B. burgdorferi s. s. (Derdáková et al. 2003).

Seropositivity of wild rodents indicates previous exposure to infected ticks (Stefancikova et al. 2004). The prevalence of anti-Borrelia antibodies was higher in Apodemus spp. than in C. glareolus (Aeschlimann et al. 1986; Vostal and Zakovska 2003; Stefancikova et al. 2004). The difference may be a result of either higher infestation of Apodemus spp. than C. glareolus by Ixodes ricinus ticks (Hanincova et al. 2003; Michalik et al. 2005) or interspecies variability.

<table>
<thead>
<tr>
<th>Rodent</th>
<th>No. of examined animals</th>
<th>No. of IgM positive</th>
<th>Prevalence (%)</th>
<th>No. of IgG positive</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clethrionomys glareolus</td>
<td>36</td>
<td>9</td>
<td>16.1</td>
<td>12</td>
<td>21.4</td>
</tr>
<tr>
<td>Apodemus flavicollis</td>
<td>34</td>
<td>10</td>
<td>29.4</td>
<td>12</td>
<td>35.3</td>
</tr>
<tr>
<td>Apodemus sylvaticus</td>
<td>11</td>
<td>2</td>
<td>18.2</td>
<td>2</td>
<td>18.2</td>
</tr>
<tr>
<td>Pitymys subterraneus</td>
<td>2</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Microtus agrestis</td>
<td>3</td>
<td>3</td>
<td>100</td>
<td>1</td>
<td>33.3</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>26</td>
<td>24.5</td>
<td>27</td>
<td>25.5</td>
</tr>
</tbody>
</table>
in the immune response (Kurtenbach et al. 1998a).

In summary, we found that muscle tissue from approximately one-sixth of the 293 animals studied was infected with *B. burgdorferi* s.l. Most infections were caused by *B. afzelii*, although infection with *B. burgdorferi* s. s. was also quite frequent. Infection with *B. burgdorferi* s. l. was more common in bank voles than in wood mice or yellow-necked mice. The prevalence of anti-*Borrelia* antibodies was higher in wood mice and yellow-necked mice than in bank voles. We conclude that small wild rodents in the Czech Republic can serve as hosts for *B. burgdorferi* s. l.

**ACKNOWLEDGMENTS**

The authors thank M. Pejcoch for rodent capture, G. Lipinova for technical assistance, and A. Nemec and J. Kybic for helpful comments. This work was supported in part by grant NR/8263-3 from the Internal Grant Agency of the Ministry of Health of the Czech Republic.

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Chapter 3: *Borrelia burgdorferi* s. 1. in wild rodents


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Chapter 4

Detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato in dogs in the Czech Republic.

Chapter 4: Detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in dogs

Detecting *Anaplasma phagocytophilum* and *Borrelia burgdorferi* Sensu Lato in Dogs in the Czech Republic

Katerina Kybicová,¹ Pavel Schánilec,² Dagmar Hulínská,¹ Lenka Uherková,¹ Zuzana Kurzová,¹ and Sandra Spejchalová²

Abstract

The aim of this study is to present molecular, serologic, and clinical findings for dogs that were naturally infected with *Anaplasma phagocytophilum* or *Borrelia burgdorferi* sensu lato (s. l.) in the Czech Republic. This data can provide information relevant to human infection. In total, blood samples from 296 dogs and 118 engorged ticks were examined. Samples were tested for *A. phagocytophilum* using polymerase chain reaction (PCR) amplification, nested PCR, and direct sequencing of the 16S rDNA, and for *B. burgdorferi* s. l. using PCR amplification of the 16S rDNA and restriction fragment length polymorphism analysis of the 5S-23S rDNA intergenic spacer. In addition, blood samples were screened for antibodies to these bacteria. Ten (3.4%) dogs were PCR-positive for *A. phagocytophilum*. Morulae of *A. phagocytophilum* in granulocytes were found in two of these dogs. Nine of the PCR-positive dogs had clinical signs related to anaplasmosis. Statistically significant differences in the PCR detection rates were found between breeds and between symptomatic and asymptomatic dogs. Infection with *Borrelia garinii* was detected by PCR in a dog with meningoencephalitis. DNA of *A. phagocytophilum* and *B. burgdorferi* s. l. (*B. garinii* or *Borrelia afzelii*) was detected in 8.5% and 6.8% of ticks, respectively. Immunoglobulin (Ig) G seropositivity to *A. phagocytophilum* was 26%. Significant differences were found with respect to breed and gender. IgM and IgG antibodies to *B. burgdorferi* s. l. were detected in 2.4% and 10.3% of dogs, respectively. Our findings suggest that the exposure to *B. burgdorferi* s. l. exists in dogs in the Czech Republic, and exposure to *A. phagocytophilum* is common.

Key Words: *Anaplasma*—Arbovirus(es)—*Borrelia*—Diagnostics—Ixodes—Lyme disease—Tick(s). Vector Borne Zoonotic Dis. 0, 000–000.

Introduction

Dogs are important domestic hosts of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato (s. l.). Data on vector-borne infections in dogs can provide important information for the potential of human infection in a particular geographic location (Duncan et al. 2005). Both *A. phagocytophilum* and *B. burgdorferi* s. l. are transmitted by ticks of the genus *Ixodes* (Parola and Raoul 2001).

*A. phagocytophilum* is a Gram-negative obligate intercellular rickettsial bacterium found in neutrophil granulocytes (Parola and Raoult 2001). *A. phagocytophilum* is known to cause granulocytic anaplasmosis in humans and domestic animals such as dogs, horses, cattle, sheep, goats, llamas, and cats (Engvall et al. 1996, Barlough et al. 1997, Bjøersdorff et al. 1999, Engvall et al. 2002, Skotarczak 2003, Lester et al. 2005, Postout et al. 2005, Lillini et al. 2006). Anaplasmosis signs include fever, fatigue, inappetence, lethargy, lameness, and gastrointestinal and central nervous system signs (Rikihisa et al. 1991, Greig et al. 1996, Engvall et al. 1997, Engvall et al. 2002). The related hematologic and biochemical abnormalities are anemia, thrombocytopenia, lymphopenia, and elevated serum alkaline phosphatase activity (Greig et al. 1996, Goldman et al. 1998). Epidemiologic studies of *A. phagocytophilum* in dogs using polymerase chain reaction (PCR) and serologic methods are available in the literature for various countries. Only case reports are available in the Czech Republic. Two cases of granulocytic anaplasmosis in dogs in the Czech Republic were described by Huml et al. (1996), one case by Melter et al. (2007), and four cases by Spejchalova et al. (2008). *A. phagocytophilum* was identified also in horses and cows by Hulínská et al. (2004).

Lyme disease (borreliosis) is a zoonotic, tick-borne disease caused by a spirochete *B. burgdorferi* s. l., which actively mu-
grates in body tissues. The clinical form of borreliosis occurs in humans and domestic animals, especially dogs, horses, and cattle (Burgess et al. 1987, Greene et al. 1991, Cohen et al. 1992). Canine borreliosis most commonly affects the limb joints (Skotarczak and Wodecka 2003, Skotarczak et al. 2005), with clinical manifestations such as arthritis and arthralgia (Jacobson et al. 1996). Other associated signs are malaise, fever, inappetence, fatigue, and lameness. Even though the signs are the same as in humans, they are more difficult to detect in dogs and develop in relatively few of them (Levy and Magnarelli 1992). Serologic reactivity to B. burgdorferi s. l. in dogs was analyzed in the Czech Republic (Pejchalová et al. 2006). B. burgdorferi s. l. infection prevalence in questing ticks from Czech Republic were found by Pejchalová et al. (2007) to be 12.1%. A. phagocytophilum prevalence in ticks (14.7%) was suggested by Skitová et al. (2007); however, less specific primers EHR521/747 were used.

Data on the prevalence of infection with A. phagocytophilum and B. burgdorferi s. l. in dogs in the Czech Republic is lacking, except for the above-mentioned serologic study. In the present study, we attempt to gather molecular, serologic, and clinical findings for dogs that were naturally infected with A. phagocytophilum or B. burgdorferi s. l. in the Czech Republic.

Material and Methods

Study sites and animals

All dogs examined in the Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic and in the Veterinary Clinic in Jablonec nad Nisou, Czech Republic between November 2005 and October 2007 were included in the study. We also included dogs of the clinic students and employees and 39 hunting dogs examined for a different study. The 292 dogs came from various locations in the Czech Republic, principally from South (202) and North Moravia (32) and from North Bohemia (62).

None had traveled outside of the country during the 6 months prior to presentation. Data on the age, gender, breed, geographical origin, health status, and purpose (working dog or pet dog) were recorded.

The dogs were divided into two groups. Group A consisted of dogs showing clinical signs attributable to infection with A. phagocytophilum or B. burgdorferi s. l. The inclusion criteria for group A was the presence of any of the following signs: apathy, fever, lameness, lethargy, inappetence, or gastrointestinal and central nervous system disorders. Dogs of group B were healthy or without a diagnosis or signs not attributable to the studied infections such as trauma or epilepsy. Samples from dogs were collected on the day of presentation to the clinic.

Blood was drawn from the jugular or cephalic vein. Buffy-coat blood smears were made and immediately stained by the Giemsa method. Ethylenediaminetetraacetic acid-anticoagulated whole blood and serum samples were taken from each dog. Attached engorged ticks were removed using forceps, identified by species and life stage, and stored individually in microcentrifuge tubes at 2 to 8°C prior to DNA extraction.

DNA extraction

Total DNA was isolated from blood samples with a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany): 200 μL of blood was transferred to a tube containing 200 μL of lysis buffer and 40 μL of proteinase K, mixed, lysed at 56°C for 1 h, and continued according to manufacturer’s protocol. Purified DNA was stored at -20°C before using it as a template for PCR amplification.

Ticks were washed in phosphate-buffered saline solution before DNA extraction using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). Ticks were transferred to a tube containing 180 μL of lysis buffer and 20 μL of proteinase K, crushed with a sterile scalpel, mixed, and incubated at 56°C for at least 2 h.

PCR amplification

All samples were analyzed by standard PCR with the Ehr 521-790 primer set (for Anaplasma) (Pancholi et al. 1995) and the LD primer set (for Borrelia) (Marconi and Garon 1992), both targeting the 16S rDNA. PCR amplification was performed in a Peltier Cycler (MJ Research, Waltham, MA, USA). The reaction mixture consisted of 2.5 μL of DNA extract as a template and 1 μM solution of each primer (Biotec, Czech Republic) in a total volume of 25 μL.

If the primary PCR products were separated by electrophoresis in 1% agarose gel and stained with ethidium bromide. Previously extracted DNA of B. garinii strain 310M was used as a positive control. Purified water served as a negative control.

 Nested PCR for Anaplasma detection

The samples found PCR positive for Anaplasma were retested by nested PCR with two sets of primers targeting also the 16S rDNA gene (ge3a, ge10r and ge9f, ge2) (Massung et al. 1998). It has been demonstrated that nested PCR with these primers and standard PCR with the Ehr 521-790 primers have the same detection limits (Massung and Slater 2003). The primary reaction mixture used 2.5 μL of DNA extract and 0.5 μM solution of each primer in a total volume of 25 μL Hot Start Master Mix. Cycling conditions involved an initial 15 min denaturation at 95°C, 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 60 sec at 72°C and a final extension of 5 min at 72°C. The reaction mixture for the nested amplifications used 0.5 μL of the primary PCR product as the template and 0.2 μM solution of each primer in a total volume of 25 μL. The nested cycling conditions were the same as those for the primary amplification, except that only 30 cycles were run. DNA of A. phagocytophilum of an infected dog was used as a positive control.

DNA sequencing

To prove that the positive PCR results were truly due to A. phagocytophilum, all positive PCR products from nested PCR amplification were directly analyzed on a CEQ 2000XL sequencer (Beckman Coulter, Buckinghamshire, UK). Size of targeted DNA sequence was 497 bp without primer-annealing areas.
Restriction fragment length polymorphism analysis for Borrelia detection

The Borrelia-positive DNA samples were tested by restriction fragment length polymorphism (RFLP) analysis with SS (rrfA)-235 (rrfB) rDNA intergenic spacer primers (222–255 bp) (Derdakova et al. 2003) using the same procedure as in Kybicová et al. (2008).

SeroLOGY, INDIRECT IMMUNOFLOURESCENT ASSAY, AND ENZYME-LINKED IMMUNOSORBENT ASSAY

Indirect immunofluorescence assay (IFA) for the detection of canine immunoglobulin (Ig) G antibodies against A. phagocytophilum (Fuller Laboratories, Fullerton, CA) was used according to the manufacturer's instructions. Examination of the slides was performed using a fluorescence microscope at 400-fold magnification. The fluorescence intensity at a dilution of 1:640 was used as the cutoff level. A positive reaction was manifested by apple green fluorescence of inclusion bodies (morulae).

The sera were also examined by an enzyme-linked immunosorbent assay (ELISA) (Test-line, Brno, Czech Republic) for detection of specific antibodies against B. burgdorferi s. l. in dogs in the IgG and IgM class (against antigens: OspA, OspC, p41, p100) according to the manufacturer's instructions, with serum dilution 1:400.

BUFFY COAT SMEARS

Buffy coat smears were stained with Giemsa and observed under 1000-fold magnification. For each Anaplasma-positive dog found by PCR, one buffy coat smear was examined for the presence of morulae.

Statistical analysis

All statistical tests were performed with respect to breed, age group, purpose (working dog or family dog), gender, and group A/B (symptomatic/asymptomatic). For statistical analysis, the dogs were divided into five age groups (≤1, 2–4, 5–7, 8–10, ≥11 years) according to their age after the onset of clinical signs. Breeds were separated according to FCI groups (Federation cynologique internationale, www.fci.be).

To obtain a sufficiently large sample in each group for statistical analysis, cross-breeds were considered jointly with FCI group V, and also group VI with group VII, and group IX with group X. The Fisher exact test was used to compare PCR, IFA, and ELISA results. The chi-square test was used for analysis of differences between antibody titers. Multivariate analysis using logistic regression was used for analysis of PCR and IFA results with respect to all parameters together except breeds, because of a limited number of samples. Values of p < 0.05 were considered significant, and odds ratios and 95% confidence intervals are reported. Calculations were realized in the Stata program (StataCorp, College Station, TX).

Results

DOGS

The study group included 296 dogs, 131 females and 165 males, mean age 6.5 years, age range 2 months to 16 years. Their distribution by age, gender, breed, and purpose is shown in Table 1. The group was divided into two subgroups: group A, 141 symptomatic dogs, and group B, 155 asymptomatic dogs. Frequent hematologic abnormalities in group A (symptomatic) were anemia, thrombocytopenia, and leukocytosis. In group B, 55 dogs were clinically healthy and 100 had a neurologic (epilepsy) or oncologic diagnosis, unrelated to infection. None of the dogs of group B showed hematologic or biochemical abnormalities.

Molecular detection of A. phagocytophilum

DNA of A. phagocytophilum was detected by PCR in 10 (3.4%) of 296 dogs. Of these dogs, nine belonged to group A (9/141, 6.4%) and one to group B (1/155, 0.6%) (Table 2); the difference between groups was statistically significant (odds ratio 11.8, confidence interval 4.4-96.9, p = 0.02 by logistic regression). Six infected dogs were terriers and four were retrievers (FCI groups III and VIII), with the difference between breeds being significant (p = 0.001). Eight of the 10 PCR-positive dogs were diagnosed between April and July, one dog was diagnosed in August, and one in September.

Clinical sings and diagnosis of the nine PCR positive dogs in group A were fever (3), apathy (2), inappetence (1), pyometra (1), gastroenteritis (2), and seizure (2). Hematologic abnormalities included anemia (4), thrombocytopenia (2), leukocytosis (2), neutropenia (2), neutrophilia (3), lymphopenia (2), monocytosis (1), eosinopenia (1), and leukopenia (1). Biochemical abnormalities such as elevated alkaline phosphatase (3) or alanine aminotransferase (1), hyperbilirubine-

<table>
<thead>
<tr>
<th>Breed</th>
<th>Group A (n = 141)</th>
<th>Group B (n = 155)</th>
<th>Total (n = 296)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (intact)</td>
<td>50</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Female (spayed)</td>
<td>15</td>
<td>16</td>
<td>31</td>
</tr>
<tr>
<td>Male (intact)</td>
<td>71</td>
<td>86</td>
<td>157</td>
</tr>
<tr>
<td>Male (castrated)</td>
<td>5</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>≤1</td>
<td>15</td>
<td>16</td>
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<td>2–4</td>
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<td>5–7</td>
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</tr>
<tr>
<td>8–10</td>
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<td>≥11</td>
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</tr>
<tr>
<td>FCI I</td>
<td>11</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>FCI II</td>
<td>19</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>FCI III</td>
<td>22</td>
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<td>33</td>
</tr>
<tr>
<td>FCI IV</td>
<td>10</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td>FCI V + cross-breeds</td>
<td>22</td>
<td>25</td>
<td>47</td>
</tr>
<tr>
<td>FCI VI + FCI VII</td>
<td>6</td>
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</tr>
<tr>
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<td>32</td>
<td>71</td>
</tr>
<tr>
<td>FCI IX + FCI X</td>
<td>12</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>Purpose</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Working dog</td>
<td>71</td>
<td>99</td>
<td>170</td>
</tr>
<tr>
<td>Family dog (pet)</td>
<td>70</td>
<td>56</td>
<td>126</td>
</tr>
</tbody>
</table>

Group A is symptomatic dogs and group B is asymptomatic dogs. FCI, Federation cynologique internationale.
mialgia (1), hypoproteinemia (1), hyperglycemia (1) were observed.

Sequencing of the 10 positive PCR products revealed the gene sequence of *A. phagocytophilum*. The sequences of the isolates were submitted to the NCBI database with the GenBank accession numbers: EU847526 to EU847535.

**Molecular detection of *B. burgdorferi* s. l.**

DNA of *B. burgdorferi* s. l. was only found in one dog of group A, an 8-year-old labrador retriever. The dog was diagnosed with lymphocytic meningoencephalitis in April 2006. We found no biochemical and hematologic abnormalities in blood, except for a slight anemia. Pleocytosis with small and activated lymphocytes was detected in the cerebrospinal fluid. Using RFLP, the agent was identified as *Borrelia garinii*.

**Ticks**

A total of 118 engorged *I. ricinus* adult ticks (106 females, 12 males) were collected from 67 dogs of groups A and B (42 and 76 ticks, respectively) and individually screened for the presence of DNAs of *A. phagocytophilum* and *B. burgdorferi* s. l. Ten ticks (8.5%), all females, were infected with *A. phagocytophilum*. Three of these 10 positive ticks were collected from two *Anaplasma*-positive dogs. *Borrelia* DNA was found in eight ticks (6.8%), seven females and one male. All infected ticks originated from *Borrelia*-negative dogs. *B. garinii* was detected in five ticks and *Borrelia afzelii* in three ticks, using RFLP.

**Serologic findings**

Using IFA, IgG antibodies to *A. phagocytophilum* were detected in 77 dogs, which corresponds to an overall seroprevalence rate of 25.9%. There was no significant difference between groups A and B with 39 (27.5%) and 38 (24.7%) seropositive dogs, respectively. Similarly, there was no significant difference between groups A and B with respect to antibody titers. Very high antibody titers (1:1280) were similarly frequent in both groups, found in 17 of 141 group A dogs and in 17 of 155 group B dogs. Five of the 10 PCR-positive dogs were seropositive, with antibody titers of 1:2560 (n = 1), 1:1280 (n = 2), and 1:640 (n = 2). There was a statistically significant difference in seropositivity between breeds (p = 0.002). The highest seropositivity rates were found in FCI groups II and VIII; males showed higher seropositivity.

---

<table>
<thead>
<tr>
<th>TABLE 2. PCR, IFA, AND ELISA POSITIVITY RESULTS WITH RESPECT TO THE PRESENCE OF SIGNS, GENDER, AGE, BREED, AND DOG PURPOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anaplasma phagocytophilum</strong></td>
</tr>
<tr>
<td>----------------------------------</td>
</tr>
<tr>
<td><strong>No. of PCR-positive dogs</strong></td>
</tr>
<tr>
<td><strong>No. of IFA (IgG)-positive dogs</strong></td>
</tr>
<tr>
<td><strong>No. of ELISA (IgG)-positive dogs</strong></td>
</tr>
<tr>
<td>Group A (symptomatic)</td>
</tr>
<tr>
<td>Group B (asymptomatic)</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Female (intact)</td>
</tr>
<tr>
<td>Female (spayed)</td>
</tr>
<tr>
<td>Male (intact)</td>
</tr>
<tr>
<td>Male (castrated)</td>
</tr>
<tr>
<td>Age (y)</td>
</tr>
<tr>
<td>≤1</td>
</tr>
<tr>
<td>2–4</td>
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<tr>
<td>5–7</td>
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<tr>
<td>8–10</td>
</tr>
<tr>
<td>≥11</td>
</tr>
<tr>
<td>Breed</td>
</tr>
<tr>
<td>FCI I</td>
</tr>
<tr>
<td>FCI II</td>
</tr>
<tr>
<td>FCI III</td>
</tr>
<tr>
<td>FCI IV</td>
</tr>
<tr>
<td>FCI V + cross-bredse</td>
</tr>
<tr>
<td>FCI VI + FCI VII</td>
</tr>
<tr>
<td>FCI VIII</td>
</tr>
<tr>
<td>FCI IX + FCI X</td>
</tr>
<tr>
<td>Purpose</td>
</tr>
<tr>
<td>Working dog</td>
</tr>
<tr>
<td>Family dog (pet)</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; IFA, immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; FCI, Federation cynologique internationale.
rates than females (odds ratio 2.8, confidence interval 1.52-5.13, p = 0.001 by logistic regression, Table 2). Using ELISA, IgM and IgG antibodies to B. burgdorferi s. l. were detected in 7 and 30 dogs, respectively (Table 2). The one PCR-positive dog was found negative in both IgM and IgG. Thus the overall seroprevalence rates were 2.4% and 10.3%, respectively. There was no significant difference in seroprevalence between groups A and B or with respect to other studied parameters. The number of dogs with IgG antibodies to both A. phagocytophilum and B. burgdorferi s. l. was 13 (4.4%).

**Buffy coat smears**

By examination of buffy coats, morulae of A. phagocytophilum (Fig. 1) were detected in peripheral blood neutrophil granulocytes of 2 of the 10 PCR-positive dogs of group A, mostly at a rate of one morula per infected neutrophil.

**Discussion**

The seropositivity rates in the examined dogs indicate natural exposure to A. phagocytophilum and B. burgdorferi s. l. We found a 25.9% positivity of IgG antibodies against A. phagocytophilum. For comparison, the reported canine seroprevalence rates were 17.7% for granulocytic Ehrlichia in Sweden (Egenvall et al. 2002) and 7.5% for A. phagocytophilum in Switzerland (Pusterla et al. 1998). In Germany, antibodies to A. phagocytophilum were found in 43.2% of examined dogs (Jensen et al. 2007), in Israel in 9% (Levi et al. 2006), and in the United States, the canine seroprevalence rates were between 9.4% (Magnarelli et al. 1997) and 29% (Beall et al. 2008). The canine seroprevalence was reported to be 11.5% and 15.5% in Spain (Solano-Gallego et al. 2006, Amusategui et al. 2008) and 34.4% in Italy (Torina and Caracappa 2006). All studies used IFA, except Beall et al. (2008), who used ELISA. The above differences may have resulted from the use of different selection criteria for examined dogs. In the present study, no difference in seropositivity was found between symptomatic (27.5%) and asymptomatic (24.5%) dogs. Similar findings have been reported in Germany and the United States (Jensen et al. 2007, Beall et al. 2008). In our study, seropositivity in dogs without clinical signs can be explained by persistent antibodies as reported in chronically infected animals (Egenvall et al. 2008a). Seropositivity in many healthy dogs indicates that antibodies to A. phagocytophilum in dogs can persist (Madigan et al. 1990, Magnarelli et al. 1997, Engvall et al. 2002).

In the present study, DNA of A. phagocytophilum was detected in 3.4% (10/296) of dogs. Similar PCR positivity rates (i.e., 6.3%, 5.5%, and 9.5%) have been reported in Germany (Jensen et al. 2007), Italy (Torina and Caracappa 2006), and the United States (Beall et al. 2008), respectively. One out of 155 healthy dogs was PCR positive for A. phagocytophilum, which is similar to the study of Beall et al. (2008) in the United States (7/222 healthy dogs). Morulae in peripheral blood granulocytes were observed in only two of 10 PCR positive dogs, which corresponds to the findings of Jensen et al. (2007) (2/6). The inclusion bodies produced by A. phagocytophilum appear in granulocytes and can be demonstrated by microscopy only at the peak of the acute infection, which usually lasts only a few days (Engvall et al. 2002).

We found 2.4% IgM and 10.3% IgG canine seropositivity rates for B. burgdorferi s. l. Serologic detection of B. burgdorferi s. l. in dogs in the Czech Republic performed in 2006 (Pechalova et al. 2006) showed a seroprevalence rate of 6.5%. The reported figures vary widely from 0.6% and 6.9% in Spain (Solano-Gallego et al. 2006, Amusategui et al. 2008*), 3.9% in Sweden (Egenvall et al. 2000b*), and 1.85% in Canada (Gary et al. 2006) to 11% in the United States (Beall et al. 2008), approximately 20% in France, the United Kingdom, and Denmark (Doby et al. 1988*, May et al. 1991, Hansen and Dietz 1989*), 40.2% in Poland (Skotarczak et al. 2005).

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![FIG. 1. Morulae of *A. phagocytophilum* in neutrophil granulocytes of a dog.](image-url)
and 50% in Slovakia (Stefanciková et al. 1998). Most of the studies were based on ELISA, except for those marked by an asterisk (*), which were based on IFA. As for *Anaplasma phagocytophilum*, the differences may have resulted from different dog selection criteria. Differences in canine seroprevalence rates for tick-borne diseases can arise from variability in tick densities or proportion of infected ticks.

In the present study, we detected DNA of *Borrelia burgdorferi* s.l. in peripheral blood of only one PCR-positive dog. This low yield can be explained by the transient nature of the presence of the spirochetes in the blood (Straubinger et al. 1997, Chang et al. 2001). Using PCR, *Borrelia* DNA can only be detected in blood in the early stage of infection (Skotarczak and Wodecka 2003). Once the microorganism is disseminated in the body, a variety of organs can be affected, especially the skin, joints, heart, and central and peripheral nervous systems (Straubinger et al. 1997).

We found DNA of *A. phagocytophilum* and *B. burgdorferi* s.l. in 8.5% and 6.8% of ticks, respectively. *Anaplasma*-positive ticks on positive dogs might have become positive during feeding. Similar findings have been reported in Poland (Zygner et al. 2008). We found no coincidence of *Anaplasma* and *Borrelia* in dogs or in ticks.

In conclusion, our findings suggest that exposure to *A. phagocytophilum* is common in dogs in the Czech Republic. This study demonstrated that symptomatic dogs from North and South Moravia and from North Bohemia had higher chance to be infected with *A. phagocytophilum* that asymptomatic dogs. On the other hand, no correlation was found between clinical signs of anaplasmosis or borreliosis and positive antibody titers to *A. phagocytophilum* or *B. burgdorferi* s.l., respectively.

**Acknowledgments**

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**Disclosure Statement**

No competing financial interests exist.

**References**


Chapter 4: Detection of *A. phagocytophilum* and *B. burgdorferi* in dogs


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Chapter 5

Clinical and Diagnostic Features in Three Dogs Naturally Infected with Borrelia spp.

Clinical and Diagnostic Features in Three Dogs Naturally Infected with Borrelia spp.

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Abstract

The aim of this study is to present clinical and neurological signs, laboratory abnormalities, serologic and/or molecular findings in three dogs from the region of Brno in Czech Republic. All dogs were naturally infected with Borrelia burgdorferi sensu lato. The evidence of borrelial infection was proved by serial blood sampling for IgM and IgG anti-borrelial antibodies or plasma PCR. The dogs manifested corresponding clinical signs and one or more of the following criteria were fulfilled: (1) 4-fold or greater increase or decrease in B. burgdorferi s. l. IgM or IgG antibodies serial titres in acute and convalescent stage of infection, (2) a shift from positive IgM to IgG antibodies titres, (3) decrease of IgM with concurrent increase of IgG antibodies in serial titres, (4) detection of borrelial DNA by PCR. Other possible tick-borne infections were excluded. All three dogs showed neurological signs (two of them meningoencephalomyelitis, one seizure connected with progressive renal disease). Their history, clinical signs, diagnostic procedures and treatment are described. Two of dogs died and only one with meningoencephalomyelitis survived. This article showed that borrelial infection must be considered, not only in cases with febrile and orthopaedic signs but also in many other clinical syndromes.

Keywords: borrelial infection, meningoencephalomyelitis, PCR, seizure, renal disease

Introduction

Borreliosis is a zoonotic tick-borne disease caused by a Gram-negative spirochete Borrelia burgdorferi sensu lato (B. burgdorferi s.l.) which includes complex of
genospecies, three of which are considered to be pathogenic in dogs: *B. burgdorferi* sensu stricto (*B. burgdorferi* s. s.), *Borrelia garinii* (*B. garinii*) and *Borrelia afzelii* (*B. afzelii*) (Hovius et al. 1999a; Hovius 2005; Greene and Straubinger 2006). The main European vector is the tick *Ixodes ricinus*. Many species of mammals and birds were recognized as a reservoir of *B. burgdorferi* s. l. (Gern et al. 1998; Hulinska et al. 2002; Piesman and Gern 2004). The clinical form of borreliosis occurs in human, domestic animals, especially dogs, horses and cattle (Burges et al. 1987; Greene et al. 1991; Cohen et al. 1992; Skotarczak et al. 2005; Kybicová et al. 2009). The close contact between dogs and humans, the common environment and the fact that borreliosis is emerging even in the cities can be considered as indicators for the outbreaks detection (Štefančíková et al 1998; Goossens et al. 2001). Clinical signs depend on the individual host response and vary widely developing in relatively few individuals (Levy and Dreesen 1992; Levy and Magnarelli 1992). Both in humans and dogs, the condition can cause dermatological, musculoskeletal, neurological, renal and cardiovascular signs (Greene et al. 1991; Azuma et al. 1993; Straubinger et al. 1997; Straubinger 2000; Straubinger et al. 2000; Skotarczak and Wodecka 2003; Skotarczak et al. 2005; Greene and Straubinger 2006). The diagnosis is based on the combination of several factors: epidemiology-epizootological information, clinical signs, serological tests and PCR (Straubinger 2000; Straubinger et al. 2000; Skotarczak and Wodecka 2003; Skotarczak et al. 2005; Pejchalova et al. 2006; Kybicova et al. 2009). The seroprevalence for *B. burgdorferi* s. l. in dogs was assessed in many European countries, e.g. in Slovakia (Stefancikova et al. 1998), Poland (Skotarczak et al. 2005), and Sweden (Egenvall et al. 2000). Specific antibodies to *B. burgdorferi* s. l. were detected in 6.5% of the population in the Czech Republic and the seroprevalence ranged between 0.0% and 28.6% (Pejchalova et al. 2006). In a recent study also in the Czech Republic the seropositivity of ELISA in IgM and IgG was 2.4% and 10.3%, respectively (Kybicova et al. 2009). In the Czech Republic DNA of *B. garinii* was detected in a blood sample of a dog only in one case (Kybicova et al. 2009). The aim of this work is to present three cases of dog patients with a variety of clinical signs and positive detection of borrelial infection by serological tests or PCR.

### Materials and Methods

All patients were presented to the emergency service of the Clinic of Dog and Cat Diseases of the University of Veterinary and Pharmaceutical Sciences Brno (CDCD-VFU) and hospitalized there. All dogs were regularly vaccinated with polyvalent vaccines and dewormed but none of them was vaccinated against borreliosis. All dogs suffered from severe tick infestation and all dogs came from
Laboratory criteria for assessment of borrelial diagnosis

Patients were diagnosed with borrelial infection if they manifested corresponding clinical signs and one or more of the following criteria were fulfilled: (1) 4-fold or greater increase or decrease in B. burgdorferi s. l. IgM or IgG antibodies serial titres in acute and convalescent stage of infection, (2) a shift from positive IgM to IgG antibodies titres, (3) decrease of IgM with concurrent increase of IgG antibodies in serial titres, (4) detection of borrelial DNA by PCR.

CSF examination, haematology and serum biochemistry

Complete CSF analysis was made within 30 minutes from collection (Bagley 2003; Bagley and Bohn 2003; Bohn and Bagley 2003). Cell counts were performed using the Fuchs-Rosenthal counting chamber. Blood and cerebrospinal fluid (CSF) smears were stained with Hemacolor (Merck KGaA, Darmstadt, Germany). Routine haematological examination and selected biochemical values was performed.

Serology and PCR

The sera were examined by the enzyme immunoassay (EIA) for detection of borrelial IgG and IgM antibodies (Test-line, Brno, Czech Republic), by EIA for the detection of tick-borne encephalitis virus (TBEV) Ig antibodies (Test-line, Brno, Czech Republic) and by *A. phagocytophilum* immunofluorescence (IFA) canine IgG antibody test (Fuller Laboratories, Fullerton, CA, USA), all according to manufacturer’s instructions. The DNA samples were isolated from blood and CSF and were analyzed by standard PCR (Kybicova et al. 2009). Positive DNA samples were retested by restriction fragment length polymorphism (RFLP) analysis with 5S (rrfA)-23S (rrlB) rDNA intergenic spacer primers (Derdákova et al. 2003) as in (Kybicova et al. 2008).

Case studies

Case 1 (5–7/2002)

A five-month-old dog, 13 kg, intact male Nova Scotia Duck Tolling Retriever was presented with a 2 weeks history of inability to move, general hyperesthesia and
spasticity of the muscles of the head and neck. The previous veterinarian collected blood samples (day -14) for borreliosis (Table 2) because found the dog pyretic (3 previous days) and treated him with high doses of co-amoxicillin (30 mg kg\(^{-1}\) PO BID) and non-steroidal drugs. The condition of the dog improved over the next 3 days. a week later, the dog started refusing to lie down; showing kyphosis, often remaining in a sitting position and having inability to make head and neck ventroflexion. At admission (day 0), the dog showed generalized hyperesthesia including vocalization and inability to move. The mental status alternated from depression and stupor to hysteria, accompanied by episodes of opisthotonus and myoclonia of both forelimbs. The cervical muscles were hypertonic and strong pain was elicited with mild manipulation of the neck. Cranial nerves examination revealed moderate decreased trigeminal (CN V) sensation and facial (CN VII) reactivity on the left side with absence of reactions for the same nerves on the right side. Spinal reflexes showed hyperreflexia in all of four limbs. The dog remained preferentially on the right lateral recumbency. CBC (Table 1) and antinuclear antibody test (ANA test) were sampled. Standard fluid support and treatment with co-amoxicillin (25 mg kg\(^{-1}\) IV TID) was given, together with Diazepam (single dose 1.5 mg kg\(^{-1}\) IV slowly) followed by phenobarbital (2 mg kg\(^{-1}\) IV BID -TID) to control mental status. During the first two days of hospitalization the neurological status worsened, including spastic tetraparesis, tonic seizures with mild manipulation and permanent posture in right lateral recumbency. Reactivity of cranial nerves changed: CN V and CN VII function changed to hyperreactivity on the left side with severe hyporeflexia on the right side. Furthermore, direct and indirect pupillary reflexes were decreased on the right side while normal on the left. Additional findings included severe hyperesthesia in the orbital and cervical areas and bilateral blepharospasm. a multifocal lesion involving meninges, cortex, brainstem and cervical spinal cord was suspected and collection and examination of CSF was indicated. CSF analysis showed Pandy reaction (++) and white blood cell count of 40 cells l\(^{-1}\). Cytology showed 60% of small lymphocytes, 13% of activated lymphocytes, 13% of monocytes, 13% activated monocytes up to macrophages and sporadically polymorphonuclears (PMN). Biochemistry showed proteinorhachia (TP 0.81g l\(^{-1}\)). The glucose level was normal (4.17 mmol l\(^{-1}\)). Aerobic and anaerobic cultures of CSF were negative. These findings were compatible with lymphocytic meningoencephalitis suggesting an infective etiology.

The clinical and neurological status slowly improved during the following 4 days. On day 6, the patient revealed mild disorientation, generalized symmetrical ataxia with hyperreflexia of all four limbs, lower neck and head position with moderate cervical discomfort. The general hyperesthesia and the pupilar and ocular changes persisted. The dog was able to eat and drink. Blood was
sampled for TBE (negative) and borreliosis (Table 2). Dog was discharged and owners were instructed to provide nursing care. Treatment continued with co-amoxicillin (25 mg kg⁻¹ PO BID) for the following 14 days and the dose of phenobarbital tapered for the following 3 days. A week later patient’s condition gradually worsened (lethargy, anorexia and hyperesthesia) and the owner by own initiative gave him prednisone (1 mg kg⁻¹ PO for three days) and by day 21 visited CDCD-VFU facilities. The dog was lethargic, febrile (40.3 °C), tachycardic (150 beats min⁻¹), severely hyperesthetic with vision deficits on the right. Owner declined hospitalization. Treatment was changed to azithromycin (20 mg kg⁻¹ PO SID). From Day 25, the antibiotic was substituted with cefotaximum (85 mg kg⁻¹ IV TID). Serum samples were submitted for antiborrelial antibodies (Table 2). Up to Day 34, according to the owner’s information, the dog seemed better. On day 37, the patient was re-hospitalized due to the relapse of the clinical signs (hyperesthesia, hyperextension of the pelvic limbs and cervical discomfort). On day 38, CBC and biochemistry were taken (Table 1). Serum samples for borreliosis (Table 2) and anaplasmosis also were submitted. However, during the following day the patient’s condition deteriorated and the owners requested euthanasia. Postmortem CSF analysis showed Pandy reaction (++) and white blood cell count of 110 cells l⁻¹. Cytology showed 25% of small lymphocytes, 43% of activated lymphocytes up to plasma cells, 21% of PMN, 11% monocytes up to macrophages. CSF biochemistry showed proteinorhachia (0.97 g l⁻¹) and decreased glycorhachia (1.95 mmol l⁻¹, reference range: 2.7–4.2 mmol l⁻¹). Cultures and borrelial PCR were also negative.

At necropsy, macroscopically there was thickening of dura mater on the left cerebral hemisphere and multifocally through the spinal cord. Histopathology showed chronic meningoencephalomyelitis. Multiple malacic lesions were seen in the brain and spinal cord with atrophy of ventral spinal horns and their corresponding spinal roots. Additional findings were acute hepatitis, chronic diffuse granulomatous and necrotic myocarditis.

**Case 2 (7–9/2003)**

A five-year-old, 34 kg, intact male Labrador Retriever was evaluated due to a history of two days of generalized short episodes of tonic-clonic seizures with unconsciousness. Initial clinical examination (day 0) showed mild lethargy and slight generalized ataxia. CBC and biochemistry were taken (Table 1). Serology for borreliosis was positive (IgM 2.32 - positive; IgG 0.94 - dubious). The next day the dog was hospitalized. Ultrasound examination showed bilateral hyperechogenity of the kidneys suggesting renal disease (RD), glomerulonephritis (GN) or pyelonephritis (PN). Urine was collected by cystocentesis and showed alka-
**Chapter 5: Clinical and Diagnostic Features in Dogs**

Table 1: Results of haematology and biochemistry

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference range / Units</th>
<th>Case 1 day 1</th>
<th>Case 1 day 38</th>
<th>Case 2 day 0</th>
<th>Case 2 day 44</th>
<th>Case 3 day 1</th>
<th>Case 3 day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERY</td>
<td>5.5-8.5 / 10^12 l^-1</td>
<td>4.08</td>
<td>3.61</td>
<td>4.5</td>
<td>4.71</td>
<td>5.18</td>
<td>5.68</td>
</tr>
<tr>
<td>HB</td>
<td>120-180 / g l^-1</td>
<td>100</td>
<td>83</td>
<td>107</td>
<td>119</td>
<td>115</td>
<td>124</td>
</tr>
<tr>
<td>HT</td>
<td>0.37-0.55 / l^-1</td>
<td>0.28</td>
<td>0.24</td>
<td>0.33</td>
<td>0.34</td>
<td>0.35</td>
<td>0.39</td>
</tr>
<tr>
<td>TRC</td>
<td>200-500 / 10^12 l^-1</td>
<td>232</td>
<td>186</td>
<td>-</td>
<td>-</td>
<td>227</td>
<td>365</td>
</tr>
<tr>
<td>LEU</td>
<td>6-17 / 10^9 l^-1</td>
<td>36.2</td>
<td>23.9</td>
<td>8.6</td>
<td>9.6</td>
<td>8.7</td>
<td>7.1</td>
</tr>
<tr>
<td>Bands</td>
<td>0-1 / 10^9 l^-1</td>
<td>1.09</td>
<td>1.43</td>
<td>0.34</td>
<td>0.1</td>
<td>0.17</td>
<td>0</td>
</tr>
<tr>
<td>PMN</td>
<td>3-11.4 / 10^9 l^-1</td>
<td>31.8</td>
<td>19.8</td>
<td>5.59</td>
<td>7.68</td>
<td>5.48</td>
<td>2.13</td>
</tr>
<tr>
<td>LY</td>
<td>1.4-8.0 / 10^9 l^-1</td>
<td>2.53</td>
<td>2.39</td>
<td>2.15</td>
<td>0.58</td>
<td>2.09</td>
<td>4.12</td>
</tr>
<tr>
<td>MONO</td>
<td>0.1-3.0 / 10^9 l^-1</td>
<td>0.36</td>
<td>0.24</td>
<td>0</td>
<td>0</td>
<td>0.78</td>
<td>0.71</td>
</tr>
<tr>
<td>EO</td>
<td>0.6-7.5 / 10^9 l^-1</td>
<td>0.6</td>
<td>3.61</td>
<td>0.52</td>
<td>0.1</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>TP</td>
<td>55-75 / g l^-1</td>
<td>-</td>
<td>51.2</td>
<td>67.8</td>
<td>57.7</td>
<td>63.1</td>
<td>-</td>
</tr>
<tr>
<td>ALB</td>
<td>30-47 / g l^-1</td>
<td>-</td>
<td>21.6</td>
<td>30</td>
<td>28</td>
<td>30.7</td>
<td>-</td>
</tr>
<tr>
<td>A/G</td>
<td>0.7-1.1</td>
<td>-</td>
<td>0.72</td>
<td>0.79</td>
<td>0.94</td>
<td>0.95</td>
<td>-</td>
</tr>
<tr>
<td>TB</td>
<td>0-7 / μmol l^-1</td>
<td>-</td>
<td>5.6</td>
<td>-</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CRE</td>
<td>30-120 / μmol l^-1</td>
<td>-</td>
<td>118.6</td>
<td>503</td>
<td>859.1</td>
<td>65.0</td>
<td>-</td>
</tr>
<tr>
<td>U</td>
<td>2.5-7 / mmol l^-1</td>
<td>-</td>
<td>9.18</td>
<td>39.8</td>
<td>82.4</td>
<td>3.37</td>
<td>-</td>
</tr>
<tr>
<td>GLU</td>
<td>3.3-7.0 / mmol l^-1</td>
<td>-</td>
<td>5.17</td>
<td>4.85</td>
<td>8.04</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>ALP</td>
<td>0.1-1.5 / μkat l^-1</td>
<td>-</td>
<td>3.14</td>
<td>0.3</td>
<td>0.5</td>
<td>0.60</td>
<td>-</td>
</tr>
<tr>
<td>ALT</td>
<td>≤ 1 / μkat l^-1</td>
<td>-</td>
<td>2.4</td>
<td>1.6</td>
<td>2.3</td>
<td>0.74</td>
<td>-</td>
</tr>
<tr>
<td>Ca</td>
<td>2.3-3.0 / mmol l^-1</td>
<td>-</td>
<td>2.5</td>
<td>1.64</td>
<td>1.31</td>
<td>2.14</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>0.9-2.5 / mmol l^-1</td>
<td>-</td>
<td>2.01</td>
<td>3.36</td>
<td>5.04</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>Na</td>
<td>135-155 / mmol l^-1</td>
<td>-</td>
<td>151</td>
<td>152</td>
<td>145</td>
<td>143.0</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td>3.6-5.6 / mmol l^-1</td>
<td>-</td>
<td>5.3</td>
<td>5.39</td>
<td>5.28</td>
<td>4.24</td>
<td>-</td>
</tr>
</tbody>
</table>


Table 2: Results of the serological examination for *Borrelia* sp. (Case 1)

<table>
<thead>
<tr>
<th>Index of positivity</th>
<th>Day -14</th>
<th>Day 6</th>
<th>Day 25</th>
<th>Day 34</th>
<th>Day 38</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM (result)</td>
<td>0.34 (-)</td>
<td>1.54 (+)</td>
<td>2.26 (+)</td>
<td>2.20 (+)</td>
<td>3.87 (+)</td>
</tr>
<tr>
<td>IgG (result)</td>
<td>0.14 (-)</td>
<td>0.17 (-)</td>
<td>0.27 (-)</td>
<td>0.31 (-)</td>
<td>0.70 (-)</td>
</tr>
</tbody>
</table>
Chapter 5: Clinical and Diagnostic Features in Dogs

Table 3: Results of the serological examination for Borrelia sp. (Case 3)

<table>
<thead>
<tr>
<th>Index of positivity</th>
<th>Day 2</th>
<th>Day 10</th>
<th>Day 33</th>
<th>Day 422</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM (result)</td>
<td>0.5 (-)</td>
<td>0.634 (-)</td>
<td>1.044 (+/-)</td>
<td>0.517 (-)</td>
</tr>
<tr>
<td>IgG (result)</td>
<td>0.3 (-)</td>
<td>0.562 (-)</td>
<td>0.716 (-)</td>
<td>0.869 (+/-)</td>
</tr>
<tr>
<td>PCR</td>
<td>B.g. (+)</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

Urinary, hyperstenuria, cylindruria (granular casts), crystalluria (ammonium urate 1/field), also occasional bacteriuria and mild proteinuria (+) were found. Urinary protein/creatinine ratio (Up/Uc ratio) was 3.6 (normal range: ? 1). The dog was treated initially with saline 0.9%. TBE, anaplasmosis and leptospirosis (L. icterohaemorrhagiae and L. grippotyphosa) serologies were negative. On Day 4, owner declined further hospitalization. The patient was discharged with doxycycline (10 mg kg\(^{-1}\) PO BID) initially for one week. Owner missed recommended follow-ups and came on day 44. According to him the dog was doing better excepting for mild exercise intolerance. CBC and biochemistry were checked (Table 1). Borrelial antibodies were measured (IgM 1.47 - positive; IgG 3.52 - positive). Owner did not return for the next follow-ups and by telephonic communication after 2 months he reported that the dog had died.


An eight-year-old, 40 kg, intact male Labrador Retriever was referred with 1 day history of reluctance to move and progressive ataxia, which worsened to tetraparesis (day 0). The last 2 weeks the dog was being treated because a phlegmon on the right forelimb by the referring veterinarian a received one week unknown antibiotic treatment. The day before admission he started treating him with marbofloxacin. At admission (day 1) the dog seemed to be in good body condition, he had hyperemic mucous membranes, hot spot in the neck area lasting 4 days, mild antebraehial edema on the right forelimb, enlargement of the left axilar lymph node and bradycardia (60 beats min\(^{-1}\)). Neurologically, the patient was depressed, tetraparetic with a lateralized generalized ataxia to the right, pleurothotonus to the right, and also showed signs of cervical discomfort especially with ventroflexion of the head. Although the dog fell down to the right, he was able to walk few steps without help. Cranial nerves examination showed diffuse hypersensitivity in the facial areas. The spinal reflexes were normal to increased. CBC and the biochemistry were taken (Table 1). An electrocardiography (ECG) revealed bradycardia, 1st degree atrioventricular block and prolonged QT inter-
Figure 5.1: RFLP profiles for the 5S-23S rDNA intergenic spacer of samples from dog and positive controls. Lanes 2, 3, and 4 contain positive controls for *B. afzelii*, *B. garinii*, *B. burgdorferi* s. s., respectively. In lane 1 there is positive sample from dog Case 4 corresponding to *B. garinii*. Lanes M1 contain a Wide Range DNA Marker (Sigma) and lane M2 contains a PCR 20Bp Low Ladder Marker (Sigma).

val. CSF evaluation revealed Pandy reaction (++), and white blood cell count of 99 cells/l. Cytology showed 70% of small lymphocytes, 9% of middle lymphocytes up to plasmocytes, 20% of MONO and 1% PMN. Biochemistry showed proteinorhachia (1.32 g µl\(^{-1}\)) and normal glucose value (3.3 mmol µl\(^{-1}\)). Aerobic and anaerobic cultures and also borrelial PCR were negative. Phenobarbital (2 - 4 mg kg\(^{-1}\) IV TID and then BID) was administered and the treatment with marbofloxacin (2 mg kg\(^{-1}\) IV BID) was continued. Standard fluid support was given. On day 2, ANA test, *Borrelia, Anaplasma* and TBE serology were negative, however the PCR from blood for borrelial DNA was positive (Figure 5.1).

On day 3, the neurological status worsened almost to stupor and the cervical discomfort increased; there were also hypomotility of the tongue, severe blepharospasmus on the right side and intermittent neck myoclonus. Manitol was added to the therapy (1g kg\(^{-1}\) during 20 minutes, single dose). On day 4, the dog started to eat small amounts of meal but showing intention tremor and dysmetria of the head and neck, also he was able to alternate recumbency. From day 6, the dog was able to stand up and was able to go few steps with support. The marbofloxacin was changed to enrofloxacin (5 mg kg\(^{-1}\) SC BID). On day 10,
CBC, biochemistry (Table 1), *Borrelia* serology (Table 3) were taken and the dog was discharged with mild ataxia and recommended nursing care. On day 33, the dog showed only lameness on the left forelimb and both hindlimbs. Generalized ataxia was elicited after exercise. Re-examination of CBC, biochemistry (both in reference range), tests for TBE (negative) and *B. burgdorferi* s. l. (Table 3) were done. The last follow-up, on day 422, the owners complained about a 4-month history of faecal and urinary incontinence. Neurologic evaluation revealed symptoms corresponding with syndrome of cauda equina. Lumbar X-rays were normal. Borrelial serology and PCR were negative.

**Results and Discussion**

All presented cases showed historical, clinical and diagnostic findings that strongly indicated borrelial infection. In all dogs the clinical signs started between April and July. It correlates with seasonal activities of ticks and dogs (Hovius et al. 1999b; Hovius et al. 1999c; Steere 2001; Bhide et al. 2004; Pejchalova et al. 2006; Kybicova et al. 2009). Clinical illness in infected dogs occurs 2 to 6 months after tick exposure. In humans as well as in animals the clinical symptoms are divided in three stages. Stage I (early localized infection) can be in humans presented as erythema migrans. This stage is not seen in dogs. Stage II (early disseminated infection) can affect all organ systems, and can manifest itself by musculoskeletal pains, variable neurological symptoms, cardiovascular and renal symptoms. During this stage we can often see clinical symptoms and detect antibodies dynamics. A general malaise often precedes or combines with a lameness episode and can be accompanied with moderate to high fever, anorexia and listlessness. Stage III (late persistent infection) can cause chronic symptoms, recurrent arthritis and chronic neurological disorders. (Baranton 2002; Hovius 2002; Higgins 2004; Hovius 2005; Greene and Straubinger 2006).

Diagnosis borreliosis is obtained by the presence or absence of the following factors: 1) The presence of typical clinical symptoms; 2) exclusion of differential diagnosis; 3) apparent reaction to antibiotic; 4) evident contact with a tick or living in an endemic area; 5) the presence of antibodies in the blood serum. The latter criterion has been a serious diagnostic indication, however, seropositivity determined from a single sample cannot differentiate between past and present exposure, and cannot on its own constitute a basis for diagnosis of an active infection (Egenvall et al. 2001; Bhide et al. 2004; Skotarczak et al. 2005). ELISA is considered to be sufficiently specific to be used separately for determining the dynamics of the antibody response to *B. burgdorferi* s. l. and can react in early stage of illness (Hovius et al. 1999b).

The timing of serologic examination is important in determination whether
active or past infection is responsible for the seropositivity. Early serodiagnostic results are usually negative because the immune response to *Borreliae* develops gradually. Antiborrelial antibodies IgM are produced 1 to 2 weeks after infection (Hovius et al. 1999b), correlate with onset of clinical illness, and remain elevated for 2 months (Greene and Straubinger 2006). IgG-ELISA positive titres develop within 4 to 6 weeks (Appel et al. 1993), culminate at 3 months and last 1 to 2 years after exposure (Straubinger et al. 2000; Goossens et al. 2001). Simultaneous measurements of IgG and IgM and paired-sample titres are recommended for detection of borrelial infection. False-negative antibody tests results are rare (Greene and Straubinger 2006) so clinical syndromes together with PCR and serial serology indicate a very likely borrelia induced disease. The clinical and laboratory findings of borrelial infection are described in more detail for each of the cases due to the particular variations between subjects: In Case 1, the proof of infection with *Borrelia* spp. was based on an increase of more than 10-fold in IgM antibodies in 52 days (Table 2). IgM increased from negative up to high positive titres. IgG was always negative. Titres of antiborrelial antibodies were examined five times. In Case 2, antiborrelial antibodies were examined only twice, on Day 0 and 44. a shift from IgM to IgG with a decrease in IgM was detected. In Case 3, the proof of presence of borrelial infection was based on of positive PCR sera (*B. garinii*) and clinical, neurological, CSF and ECG findings. Sera testing for antiborrelial antibodies (four times) showed only a weak response at day 33rd (Table 3). Since the dog was treated two weeks before presentation, probably with antibiotics that affected *Borreliae*, it is very possible that his immune response was delayed or reduced. The later PCR detection could be explained by insufficient initial antibiotic treatment (dose, duration), repetitive proliferation of the surviving spirochetes or re-infection (Straubinger et al. 1997). The immune response and PCR findings suggested that all three cases showed signs of early disseminated stage of borrelial infection. Variable neurological signs are described in dogs (Azuma et al. 1993; Azuma et al. 1994; Baranton 2002; Hovius 2002; Higgins 2004; Hovius 2005; Greene and Straubinger 2006). Both dogs with meningoencephalomyelitis (Cases 1, 3) were CSF evaluated and the findings corresponded to a suspected central nervous system (CNS) inflammation because of protein-cytological association. Serial sampling of CSF in Case 1 showed increased activation of mononuclear cells, their increased number and no signs of other bacterial presence.

CSF findings are not pathognomonic only for borrelial infection (Adam 1999). For both dogs the PCR of CSF was negative. It corresponds with other findings by many authors because of *Borreliae* are very easily eliminated by the immune system and/or by penetrating antibiotics. Different results are described in the literature concerning culture and PCR detection *Borreliae* in nervous system (Appel et al. 1993; Hovius et al. 1999c; Chang et al. 2001; Straubinger 2000). Histological
findings in Case 1 correspond with neurological and CSF findings. In Case 2, neurological signs were reported. The origin of seizures remained unclear. They can be part of nephro-encephalopathy, because of azotaemia or CNS borrelial infection cause irritation (Azuma et al. 1993; Azuma et al. 1994). Interestingly, Chang et al. 2001 described that experimentally infected dogs developed mild focal meningitis, encephalitis, and perineuritis but without neurological signs. These symptoms in Case 2 could probably have been caused by silent borrelial CNS infection with a transient relapse. The progressive renal failure, the moderate to severe azotemia and the significant proteinuria were detected along with IgM/IgG shift (Grauer et al. 1988; Greene and Straubinger 2006; Gerber et al. 2007). A possible preponderance of Labrador and golden retrievers to renal involvement has been described. The same authors claim that the duration of clinical renal illness is 24 hours to 8 weeks (Greene and Straubinger 2006).

The ECG changes and post-mortem findings in Cases 1 and 3, make us think that the incidence of cardiac manifestations in borreliosis could be higher than previously thought. Humans have a cardiac involvement in Lyme disease in 8-10% of adults (Nagi et al. 1996; Bateman and Sigal 2000; Nalmas et al. 2007). The natural incidence of Lyme carditis in veterinary medicine has not been yet estimated. The observed cardiovascular syndromes include presence of arrhythmia (variable degrees of atrioventricular block, supraventricular and ventricular arrhythmia Levy and Duray 1988; Nalmas et al. 2007), myocarditis, pericarditis, dilated cardiomyopathy and coronary artery disease (Gasser et al. 1999). Generally, a positive Lyme titer and accompanying cardiac signs are present and this was demonstrated in our patients. The overall prognosis of Lyme carditis is good, although recovery may be delayed and late the progressive effects of inflammation and toxins can cause heart failure (Nagi et al. 1996; Gasser et al. 1999; Cepelova 2008). The chronic diffuse granulomatous and necrotic myocardiitis found in Case 1 could suggest the cystic form of the disease (Giudice et al. 2003).

CBC revealed persistent mild hypochromic anaemia in two cases (Case 1, 2) and borderline anaemia in the Case 3. Our findings are similar to those mentioned e.g. by Breitschwerdt et al. 1994, however the majority of authors did not recorded any specific CBC changes (Greene and Straubinger 2006). Anaemia can be caused in acute stage of bacteraemia or inflammation because of bone marrow suppression or due to direct penetration of *B. burgdorferi* s.l. to bone marrow. PCR findings of *Borreliae* in bone marrow were described by Hovius et al. 1999c. In the Case 1, anaemia could have been caused because the dog was young and fast growing. Moderate leukocytosis observed in Case 1 is a nonspecific sign of inflammation; it is not possible to rule out a concurrent disease that may explain this finding.
The moderate panhypoproteinemia in the Case 1 and elevated ALT (Cases 1, 2) can be a nonspecific effect of the systemic inflammation. It could be caused by fasting, but also can be suggested as a consequence of acute hepatitis caused by Borreliae or concurrent disease. In one case acute hepatitis was verified by necropsy. The higher prevalence of *Borrelia* in liver samples in symptomatic dogs is also described (Hovius et al. 1999c; Greene and Straubinger 2006). Elevated ALP in the Case 1 could be caused by the same reason but also very probably was caused with fast growth of bones. The moderate to severe azotemia, hyperphosphatemia and hypocalcemia in Case 2 are signs of progressive renal disease and were already described above.

Commonly, concurrent tick-borne diseases can occur in infested animals (Egenvall et al. 2000; Klimeš et al. 2001; Higgins 2004; Kybicova et al. 2009). Sera of all three dogs were evaluated for borreliosis (see above), TBE and anaplasmosis with negative results, thus ruling out this possibility. The dog with signs of RD (Case 2) was also negative for leptospirosis. Two dogs (Cases 1, 3) were also tested for ANA antibodies with negative result. Lupus erythematosus is a disease that can cause a lot of clinical signs (including neurological) and the test should be done as a routine in patients showing systemic or organ inflammatory diseases. Ceftriaxone, cefotaxime, azithromycin, amoxicillin and doxycycline are thought as the most effective antimicrobial drugs against Borrelia when applied at early clinical stage of infection. In Case 1, co-amoxicillin, azithromycin and cefotaxime were administered, but the use of corticosteroids could have been a trigger for reactivation of a poorly controlled infection. The penetration of antibiotics to CNS also depends on the integrity of blood-brain barrier. It is probable that co-amoxicillin could be less effective in CNS. In Case 2, the owner aborted the administration of doxycycline, which might therefore not have been sufficiently effective. Quinolones are ineffective in treating borreliosis. There were used in Case 3 because the results of PCR were known too late (a month after sampling) and the owner declined further therapy. We suggest that supportive complementary nursing care and cage rest of patients are among the mainstays of the complex therapy of encephalitis. As showed in Case 3, it could be important for surviving despite the wrong antibiotic treatment. Duration of cage rest depends on the severity of the illness. In Cases 1 and 2, the owner finished hospitalization prematurely and the patients did not get adequate rest.
Klinické příznaky a diagnostické postupy u tří psů přirozeně infikovaných Borrelia spp.

Cílem studie je popsat klinické a neurologické příznaky, laboratorní, sérologické a/nebo molekulární nálezy u tří psů z okolí Brna (Česká republika). Všichni psi byli přirozeně infikováni Borrelia burgdorferi sensu lato a infekce byla potvrzena opakovaným vyšetřením IgM a IgG boreliových protilátek nebo pomocí PCR. Psi s klinickými příznaky splňovali jedno nebo více z následných kriterií: (1) čtyřnásobné nebo vyšší zvýšení nebo snížení titrů IgM nebo IgG protilátek proti B. burgdorferi s. l. při opakovaných vyšetřeních v akutní nebo rekonvalescentní fázi infekce, (2) změna z pozitivních IgM do pozitivních IgG titrů, (3) současné snížení IgM a zvýšení IgG protilátek, (4) nález boreliové DNA metodou PCR. Další možné infekce přenášeny klíšaty byly vyloučeny. Všichni tři psi vykazovali neurologické příznaky (dva meningoencefalitidu a jeden křeče spojené s progresivním onemocněním ledvin). Je popsána anamnéza, klinické příznaky, diagnostické postupy a jejich léčba. Dva z pacientů uhynuli a jeden pacient s meningoencefalitidou přežil. Tento článek ukazuje, že boreliová infekce musí být zvažována i v případech jiných příznaků než jsou febrilní stavy a ortopedické příznaky.

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Chapter 5: Clinical and Diagnostic Features in Dogs


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Chapter 5: Clinical and Diagnostic Features in Dogs

Chapter 6

Detection of *Anaplasma phagocytophilum* in animals by real-time polymerase chain reaction.

Detection of *Anaplasma phagocytophilum* in animals

**by real-time polymerase chain reaction**

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The aim of this study was to detect *Anaplasma phagocytophilum* in wild and domesticated animals and to identify the phylogenetic relationships of different strains of this bacterium. We adapted six published conventional methods targeting 16S fragments for real-time polymerase chain reaction. Initial screening of samples from 419 animals found 37 *Anaplasma* positives, later confirmed with several different primers and a TaqMan probe. We also performed DNA quantification and melting curve analysis. The nucleic acid of *Anaplasma* sp. was detected in a higher percentage of cases in members of the deer family, hares, bank voles and mice (12.5–15%) than in foxes, boars, cows, and horses (around 4–6%). We also performed blood analysis of cows, horses, mice, and ticks removed from animals, evaluating the presence of antibodies against granulocytic *Anaplasma* sp. Finally, we subjected 11 randomly selected PCR amplified products to direct sequencing and we constructed the corresponding phylogenetic tree with respect to the *Ehrlichia equi* sequence, homologous to the human granulocytic ehrlichiosis agent. Mutual identity of the sequencing ranged from 99% to 100%.

Key words: *Anaplasma phagocytophilum*; animals; real-time PCR; melting temperature; gene sequences.

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We investigated the frequency and distribution of *Anaplasma phagocytophilum* (formerly known as *Ehrlichia phagocytophila*), including human granulocytic ehrlichiosis (HGE) agent and *Ehrlichia equi*-related variants such as white-tailed deer agent (1, 3), in wild and domesticated animals on horse and cow pastures and animal enclosures in the same regions in the Czech Republic that we have studied previously (2). In the literature there are only a small number of studies on animal reservoirs for *A. phagocytophilum* in Europe. Knowledge of the presence of the human granulocytic ehrlichiosis agent (HGE) and the human monocytic ehrlichiosis agent (HME) in humans and animals is based on polymerase chain reaction (PCR) studies (1–3, 5–8, 10). The most frequent natural reservoirs for granulocytic *Ehrlichia* *A. phagocytophilum* seem to be *Apodemus flavicollis* mice and *Clethrionomys glareolus* voles in Switzerland (10) and white-tailed deer in the United States (3, 24). The observation that Slovenian roe deer and red deer (13) are infected...
with A. phagocytophilum in 86% of cases as determined by PCR is rather surprising considering the studies mentioned above.

Interpretation of differences between PCR results from different countries may be problematic. Massung et al. (14) showed variation in the sensitivity as well as specificity of PCR assays for A. phagocytophilum. Assay Ehr 521–790 (5), which amplifies nucleic acid (DNA) from the infected animals, was recommended for screening (15) and quantification.

We adapted conventional PCR methods (1, 3–9) so that they were more standardized and faster, using the LightCycler real-time PCR technique (RT-PCR) (Roche Diagnostics GmbH, Mannheim, Germany). This enabled us to analyze more samples (8) from more animals and to validate our previous findings (2) of human granulocytic ehrlichiosis agent in patients and animals.

There is some disagreement regarding the naming of the bacterium. Recently published studies use a new nomenclature Anaplasma phagocytophila (11) and designate Ehrlichia equi the HGE agent as subjective synonyms of Ehrlichia phagocytophila. Massung et al. (14) call this bacterium A. phagocytophilum, while others (13, 19) call it A. phagocytophilum.

To the best of our knowledge, the direct PCR sequence analysis from samples of a large number of wild animals is reported here for the first time. We succeeded in properly choosing the primers for the direct sequencing that differentiate well between the different variants of A. phagocytophilum. To analyze the relationships between these variants, we sequenced the PCR products from our samples and formed a phylogenetic tree using as a reference the sequence of E. equi. We evaluated the similarity between our sequences and sequences reported in the National Center for Biotechnology Information (NCBI) BLAST network service.

MATERIALS AND METHODS

We processed samples from three groups of animals. Group 1 – mice and bank voles, Group 2 – cows and horses, Group 3 – wild game animals. From all three groups we also collected ticks. The animals of all three groups were obtained from two localities: first, in the neighborhood of Napajedla, in Moravia, and second, close to Chlumec in east Bohemia; on horse-grounds and in enclosures.

A total of 40 yellow-necked mice (Apodemus flavicollis) and 15 bank voles (Clethrionomys glareolus) were trapped on horse-ground in both localities during the years 2002–2003. The animals were euthanatized and their blood and tissue samples from the ear and spleen were stored individually, frozen at −20°C prior to DNA extraction.

Blood samples from 55 cows and 40 horses fed on the same horse-ground were collected in plastic tubes with or without EDTA (2) and were kept frozen at −20°C.

A panel of tissue samples from wild game animals – 69 wild boars (Sus scrofa), 25 red foxes (Vulpes vulpes), 40 roe deer (Capreolus capreolus), 15 fallow deer (Dama dama), 15 red deer (Cervus elaphus), 8 European hares (Lepus europaeus), and 15 moufflon sheep (Ovis musimon) is presented in Table 1 (that also includes domestic animals, mice and bank voles). Most samples were collected during the hunting season in October and November 2002–2003 or during individual hunts in enclosures near the horse-grounds in both localities. Hunters collaborated with the National Veterinary Service Laboratory and were asked to obtain samples of spleen, heart and blood from the animals, collecting them separately. Hunters placed samples in sterile containers and kept them in ice bags until shipped to the veterinary laboratory. We excised a 4–5 mm³ piece of tissue from the interior of each organ and placed it in a numbered 1.5 ml microcentrifuge tube, refrigerated it or immediately processed it for molecular diagnostic assays.

Ticks Ixodes ricinus were recovered from infected animals, comprising nine roe deer, two fallow deer, two hares, two red foxes and six boars shot in the Moravian locality, and also from six mice, two bank voles, four cows and six horses on horse-ground in the Bohemian locality. The majority of these ticks were mainly adults and females, 26 out of 82 were not engorged, 33 were partially engorged, and the rest were fully engorged. Ticks were kept frozen until DNA extraction.

Serum specimens and indirect fluorescence assay (IFA)

A total of 135 blood specimens from 40 horses, 55 cows and 40 mice were initially screened by IFA for HGE and E. equi (MRL Diagnostics, USA) as we described previously (2). Fluorescein isothiocyanate-conjugated goat anti-horse, anti-bovine and antimouse immunoglobulin G (Sigma) was used at a 1:20 dilution as secondary antibody. Serum titration started at 1:20 dilution. Interpretation of declared IgG serum: endpoint titers of 1:64 and greater were considered positive.

DNA extraction

High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) was used...
for the isolation of nucleic acids (DNA) from blood and tissue samples. Tissue samples (predominantly samples of spleen and heart from hunting animals, ear and spleen from mice) were mechanically homogenized with a micro-pestle before addition of 200 μl tissue lysis buffer, 40 μl of proteinase K (20 mg/ml) and 5 μl of lysozyme (10 mg/ml). After incubation at 55°C for 3 h, the extraction protocol was followed as recommended by the supplier. We used alkaline lysis to extract DNA from the blood and tick samples (2).

DNA amplification and analysis

Six conventional PCR methods using different primer pairs were adapted for real-time PCR (RT-PCR). Samples from 419 animals were first screened for ehrlichial nucleic acid (DNA) by RT-PCR amplification with a group-specific primer set Ehr521–Ehr790 as previously described (3, 5). The initially RT-PCR-positive samples were retested with a primer set Ep.50r-Ep.145f (8), also with the TaqMan Ep80 probe (8), and with primer sets ECC-ECB (1, 6), nested HE3-R paired with E.equi3-IP2 (7, 9), and GER3–GER4 (4) to confirm the Anaplasma positivity of the samples and to quantify the specific DNA. The complementary DNA to Anaplasma sp. was amplified with these primer sets (Table 2) using a standardized real-time PCR (RT-PCR) protocol with the LightCycler FastStart DNA Master SYBR Green I kit and with the LightCycler FastStart DNA Master Hybridization Probes kit with the TaqMan probe (both from Roche Diagnostics, Mannheim, Germany). Primers were prepared by Generi Biotech Ltd. (DNA Synthesis Service, Czech Republic) and the TaqMan probe by TIB Molbiol (DNA Synthesis Service, Roche Diagnostics, Berlin, Germany).

### Table 1

Total number of samples extracted from different species and corresponding number of *Anaplasma phagocytophila*-positive samples using six different primer pairs

<table>
<thead>
<tr>
<th>Name of animals</th>
<th>Total number of animals</th>
<th>Number of positive Infected in first screening (%)</th>
<th>Number of positive animals detected by RT-PCR with different primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ep.50r</td>
</tr>
<tr>
<td>C. glareolus</td>
<td>15</td>
<td>2</td>
<td>13.33</td>
</tr>
<tr>
<td>A. flavicollis</td>
<td>40</td>
<td>6</td>
<td>15.00</td>
</tr>
<tr>
<td>S. scrofa</td>
<td>69</td>
<td>3</td>
<td>4.35</td>
</tr>
<tr>
<td>V. vulpes</td>
<td>25</td>
<td>1</td>
<td>4.00</td>
</tr>
<tr>
<td>C. capreolus</td>
<td>40</td>
<td>5</td>
<td>12.50</td>
</tr>
<tr>
<td>D. dama</td>
<td>15</td>
<td>2</td>
<td>13.33</td>
</tr>
<tr>
<td>C. elaphus</td>
<td>15</td>
<td>2</td>
<td>13.33</td>
</tr>
<tr>
<td>L. europaeus</td>
<td>8</td>
<td>1</td>
<td>12.50</td>
</tr>
<tr>
<td>O. musimon</td>
<td>15</td>
<td>2</td>
<td>13.33</td>
</tr>
<tr>
<td>Cow</td>
<td>55</td>
<td>3</td>
<td>5.45</td>
</tr>
<tr>
<td>Horse</td>
<td>40</td>
<td>2</td>
<td>5.00</td>
</tr>
<tr>
<td>I. ricinus</td>
<td>82</td>
<td>8</td>
<td>9.76</td>
</tr>
<tr>
<td>Total hosts</td>
<td>419</td>
<td>37</td>
<td>8.83</td>
</tr>
</tbody>
</table>

ND—not done.

### Table 2

The six variants of the real-time PCR procedure adapted from conventional PCR (references in the last column)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pair</th>
<th>Annealing in °C</th>
<th>No. of cycles</th>
<th>Specificity</th>
<th>Ap</th>
<th>Ec</th>
<th>Eq</th>
<th>Bb</th>
<th>Mean Tm (± SD in °C)</th>
<th>No. of base pair</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S</td>
<td>Ehr521-Ehr790</td>
<td>60</td>
<td>40</td>
<td>+++-+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>87.3±0.40</td>
<td>293 (3, 5)</td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>Ep.50r-Ep.145f</td>
<td>62</td>
<td>40</td>
<td>+++-+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>81.7±0.37</td>
<td>110 (8)</td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>TaqMan-Ep80</td>
<td>62</td>
<td>40</td>
<td>+++-+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>88.6±0.20</td>
<td>520 (1, 6)</td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>ECC–ECB</td>
<td>45</td>
<td>45</td>
<td>+++-+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>86.5±0.27</td>
<td>239 (7, 9)</td>
<td></td>
</tr>
<tr>
<td>16S</td>
<td>HE3-R–3-IP2</td>
<td>55</td>
<td>40</td>
<td>+++-+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>83.5±0.30</td>
<td>150 (4)</td>
<td></td>
</tr>
</tbody>
</table>

We show the gene target, the primer pair used, the annealing temperature, the number of amplification cycles chosen, and the results of the measurements: the melting temperature and its standard deviation, as well as the amplicon length found by gel electrophoresis.

The 18-ml reaction mix in a glass capillary contained 2 ml of FastStart Master SYBR Green I mix, different volumes of primers corresponding to 5 μM of each, 3 mM MgCl₂, and sterilized water for RT-PCR (Roche). We added 2 μl of template DNA from the unknown samples, the same volume of negative control (water) or positive control (DNA from HGE, cultured from patients as we described previously) to the 18 μl reaction mix. The amplification program started with denaturation at 95°C for 10 min. Annealing temperatures and numbers of cycles used are shown in Table 2. Fluorescence was measured at the end of each extension step. Melting curves were acquired by heating at 20°C/s to 95°C, cooling to 55–62°C (depending on the primers) for 20 s, and then slowly heating at 0.1 or 0.2°C/s to 95°C with continuously measured fluorescence.

A modification of the nested primer RT-PCR (20) was used with the HE3-R inner primer paired with the E. equi 3-IP2 primer (7, 9). The reaction volume of 10 μl contained 1.5 μl of FastStart Master SYBR Green I buffer, 0.5 μl of FastStart polymerase, 1.8 μl of MgCl₂ corresponding to the final concentration of 4 mM, 1 μl of each primer (5 μM). Two μl of the template DNA was added. The capillary was spun down, overlaid with 5 μl of silicone oil, and spun again. Subsequently, 10 μl of the second-round mixture containing 1 μl of each inner primer (10 μM) was added. The first round PCR was performed as denaturation at 95°C for 6 min and 40 cycles at 95°C for 5 s, 48°C for 10 s, and 72°C for 20 s. The cooled capillaries were then spun down. Second amplification started at 95°C for 2 min with 40 cycles at 95°C for 5 s, 55°C for 5 s, and 72°C for 15 s. Fluorescence melting curve analysis was performed as mentioned above.

Specific TaqMan Ep80 probe was used for detection of ehrlichial amplicons as recommended by Pusterla et al. (8). We performed electrophoresis of 7 μl of the products in 2% agarose gel with a low size marker (Sigma, D7058).

We grew granulocytic A. phagocytophilum – HGE agent in HL-60 cell cultures as we described in (2). We washed the cells (0.5 ml of culture at a density of 2×10⁵ cells/ml) with PBS, extracted the DNA, and performed serial dilutions with factor three to make the quantification. We measured the melting curve analysis from five capillaries, obtaining the mean values Tₘ, and their standard deviation for each primer pair following the supplied instructions (Roche manual). Student’s t-test was used to analyze statistical significance of the Tₘ differences, with p<0.05.

**DNA sequencing**

The direct sequence analysis was made from 11 products amplified with a GER3-GER4 primer pair and from 15 products amplified with the ECC-ECB primer pair by the dideoxy chain termination procedure using the CEQ 2000 XL automatic sequencer as we described in (2). Nucleotide sequence homology searches were made through the NCBI BLAST network service. The best corresponding sequences to our acquired ones had accession numbers AF 136712 for E. phagocytophila, strain Frankonia II; AF 136713 for strain Frankonia I; AF136714 for strain Baden; AF 482761, AF172167 for E. equi; AF189153 for HGE; AF241532 for strain from a llama (Lama glama).

Sequences were aligned and analyzed using the program CLUSTALW (http://ebi.ac.uk/clustalw/). The phylogenetic tree was generated with the software TREECON, using the Kimura algorithm to calculate the genetic distances with 1000 bootstrap replicates and the neighbor-joining method to infer the tree topology.

**RESULTS**

This study validated and completed our previous report (2) on the occurrence of human granulocytic ehrlichiosis (HGE) agent in the Czech Republic by using a more standardized LightCycler RT-PCR assay. The limits of detection of 16S rRNA assays were two infected cells as tested by use of the A. phagocytophilum template DNA from our HGE culture.

A total of 419 animals were first screened for ehrlichial DNA by RT-PCR amplification with a group-specific primer set Ehr521–Ehr790. Standard curve and amplification plot derived from a threefold dilution series of the HGE control from the screening test with Ehr521–Ehr790 primers are shown in Fig. 1. The control DNA samples of HGE had concentrations from 0.1 pg/μl to 11 pg/μl. It helped us to determine that the unknown DNA concentration in animal samples ranged from 0.1–1.2 pg/μl (see Fig. 2). Thirty-seven (8.83%) of the initially positive samples out of the 419 were retested with five different primer sets which we adapted for RT-PCR. Results are shown in Table 1. The melting temperatures obtained and the cycling conditions used are shown in Table 2.

The percentage of infected animals is summarized in Table 1. Note especially the high number of cases among the deer family (12.5–13.3%), hares (12.5%), and mice and bank voles (15% and 13.3%, respectively). The sensitivity of detection using the six different methods was similar (Table 1); there was generally less detection with the TaqMan Ep80 probe (only 24 Anaplasma-positive animals) than...
Fig. 1. The inset shows the standard RT-PCR curve (cycle number versus logarithm of concentration). The main graph contains the corresponding amplification plot (fluorescence versus cycle number). From left to right, the curves were obtained from dilutions of a specific ehrlichial DNA with concentrations 11 pg/μl; 3.66 pg/μl; 1.22 pg/μl; 0.41 pg/μl; 0.14 pg/μl. The horizontal line is a negative control. Small regression error indicates good accuracy and reproducibility of the method.

Fig. 2. Amplification plot was created from serially diluted control of HGE (as in Fig. 1) and typical samples from infected animals of various species. It indicates that the unknown concentration of DNA in animal samples ranged from 0.1 to 1.2 pg/μl.

when using the primers alone or using the nested RT-PCR. The Anaplasma DNA was found in fewer cases in boars (4.3%), which were frequently infected with other bacteria (Borrelia sp., Brachyspira hyodysenteriae, Pseudomonas fragi, etc.). Samples from red foxes were mostly negative for ehrlichial DNA; only one fox was positive. Domestic cows and horses were infected in about 5% of cases. Only 9.7% of the examined ticks were found positive. Out of the 17 adults and several nymphs of ticks from 6 trapped infected mice and 2 infected bank voles, only 3 ticks were infected. Another four infected ticks were collected from deer and one tick was collected from a horse. The tick samples show the same Tm as the samples from infected animals and the positive HGE control. Samples from different tissues of the same infected animal were always all infected.

The melting curve analysis with the primer pair Ehr521–Ehr790 used in screening was performed on 37 positive samples. In all of them
$T_m = 87.3 \pm 0.40$ was found. This is consistent with the $T_m$ from the HGE control sample (see Fig. 3). The $T_m$ temperatures obtained for the other primer pairs are reported in Table 2. Fig. 4 shows the melting curves for the Ep.50r-Ep.145f primers for the animal samples and the HGE control.

The specificity of the primers with respect to *Anaplasma phagocytophylum*, *Ehrlichia chaffeensis*, *E. equi* and *Borrelia burgdorferi* sensu lato is shown in Table 2. The primer ECC-ECB also reacted with *B. burgdorferi* sensu lato; primers Ehr521–Ehr790, Ep.50r-Ep.145f and ECC-ECB reacted with *E. chaffeensis*.

The gel electrophoresis measurements identify the length of the Ehr521–Ehr790 amplicon as 293 bp for the positive samples, which corresponds to the HGE control. This check was performed after all the RT-PCR amplifications and the lengths determined are shown in Table 2.

Blood samples of 40 horses, 55 cows, and 40 mice were screened for specific antibodies against *A. phagocytophilum* in indirect immunofluorescence antibody (IFA) tests. Antibodies...
were found in two horses, three cows and six mice. These animals were also found positive with the screening RT-PCR. Three cows and two horses had positive IFA tests with anti-HGE and anti-\textit{E. equi} antigens.

Direct microscopic examinations of Giemsa-stained buffy coat blood smears of one infected cow showed intragranulocytic inclusions (morulae) in some cells. No morulae were found in blood samples from PCR-positive horses and mice.

We performed the sequence analysis of a 520 bp (with the ECC-ECB primer pair) and 150 bp (with the GER3–GER4 primer pair) phylogenetically informative region in the 5’ end of the 16S rRNA gene. The \textit{E. equi} samples from two horses (100% homologous with HGE) (11) were 99.5% homologous to the selected samples from other animals (two roe deer, one red deer, one fallow deer, one mouflon, one red fox, one boar, one bank vole and one tick). The sequences from these animal samples were 99.6−99.8% homologous to the \textit{A. phagocytophilum} sequence from the NCBI BLAST database. There were 2−6 nucleotide differences between the animal pathogen amplicon sequences and the published sequence for HGE. The corresponding phylogenetic tree obtained using the software package CLUSTALX and TREECON is shown in Fig. 5. The tree shows that the ehrlichial agents in the wild and domesticated animals from the Czech Republic belong to the \textit{A. phagocytophilum} group. Four \textit{A. phagocytophilum} 16S rRNA genogroup variants were sequenced: variant Baden, variant Frankonia I, variant Franconia II, and variant \textit{Llama ehrlichia} from \textit{Lama glama} (22).

**DISCUSSION**

The reported prevalences of infection of \textit{I. ricinus} ticks with \textit{A. phagocytophilum} and HGE were 0.8% in Switzerland (10, 16), 3.2% in Slovenia (12), 3.1 and 9.2% on the east and west coasts of Sweden (4), and 24.4% in a region of Italy. These prevalences correspond with the results from our study – we proved DNA of \textit{A. phagocytophilum} in 9.76% of cases in ticks collected on animals in east Bohemia and in Moravia. The amount of \textit{Anaplasma} DNA in ticks depends on many factors: the tick's nutrition status, the stage of the tick, the time of year, the geographical region, and the type of hosts.

Our findings of 13.3−15% infection with ehrlichial DNA in samples from bank voles \textit{C. glareolus} and yellow-necked mice \textit{A. flavicollis} are comparable with findings in Switzerland.
Chapter 6: Detection of *A. phagocytophilum* by RT-PCR

(10) and in the United Kingdom (19). We found that the percentage of *Anaplasma*-positive animals was significantly higher in mice (15%) than in bank voles (13.5%). Bank voles and mice were known to host larval *I. ricinus* tick. We found 17 adult ticks and several nymphs on 6 infected mice and 2 infected bank voles, but only 3 of the ticks hosted *A. phagocytophilum*.

Our findings confirm that wild animals such as deer and mice were infected with a similar genotype of *A. phagocytophilum*, as were ticks, cows and horses (9). We detected DNA of *A. phagocytophilum* in a higher percentage of cases in members of the deer family, hares, bank voles and mice (12.5–15%) than in foxes, boars, cows and horses (around 4–6%). Our results from RT-PCR are comparable with our last examination of the HGE agent made with conventional PCR (2) and with observations in Europe (10) and in the United States (3, 5, 7), where HGE is endemic.

Reaction results with a TaqMan probe Ep80 and melting temperatures with five different primer pairs were similar for HGE and *E. equi* (16, 17). The sensitivity of detection of specific ehrlichial DNA in samples of infected animals using the six different methods was similar. We found that in naturally infected animals there was generally less detection with the Taq Man Ep80 probe than when using the primers alone, contrary to the results for animals infected purposefully (8).

Differences in the *T*~m~ value between HGE and variants of *A. phagocytophilum* in different RT-PCR assays were so small that they could not be used for distinguishing these variants. However, our results indicate that the fluorescence melting curve analysis of PCR products can be used for rapid detection of these agents (16).

The direct sequence analysis of the 16S rRNA can be used for the detection of closely related *Anaplasma* species in humans and in animals (17, 24). The study from Slovenia indicates (12) that the *groESL* gene should be sequenced in order to answer the question about reservoir hosts (18, 19). Sequence comparison of the 16S rRNA gene is recognized as one of the powerful methods for determining the phylogenetic relationships of bacteria as shown by Wen et al. (17). In the nested RT-PCR reaction the ECC-ECB primer pair produced a 390 bp amplicon and the GER3-GER4 primer pair produced a 150 bp amplicon, containing the species-specific signature sequences. The choice of these amplicons for direct sequencing allowed us to differentiate between the bacterial species. The phylogenetic tree that we constructed classifies the ehrlichial agents obtained from animals that were most closely related to the *A. phagocytophilum* genogroup.

Some authors (10, 12, 17) showed a high degree of homology of partial 16S rRNA gene sequences of granulocytic ehrlichia *A. phagocyto-

philum* from ticks, rodents and deer with the HGE agent isolated from humans. Others (11, 14, 15, 21) found four variants differing in two nucleotides from the HGE or *E. equi*. They reported that more than one strain of *A. phagocyto-

philum* exists both in the United States and in Europe. We found differences between sequences of ehrlichial DNA extracted from a deer, boar, red fox, bank vole, tick and two horses of only 2–6 nucleotides.

We thank the 15 hunters for providing part of the sample material, J. Votyppa, Dr. J. Plch and Dr. Z. Kurzova for technical assistance with PCR and IFA tests, Dr. Č. Vlcek for assistance with the sequencing machine, Ing. Brada for help with statistical methods.

REFERENCES


Chapter 6: Detection of A. phagocytophilum by RT PCR


Chapter 7

Discussion

We studied the occurrence of two tick-borne bacteria, B. burgdorferi s. l. and A. phagocytophilum in their main vector in the Czech Republic, tick I. ricinus. Ticks were collected from domestic hosts - dogs, horses and cows - as well as from wild animals - deer, hares, foxes, boars, mice and bank voles.

In ticks collected from dogs in north Bohemia and Moravia, we found (Kybicová et al. 2009) the DNA of A. phagocytophilum and B. burgdorferi s. l. in 8.5% and 6.8% of cases, respectively. Anaplasma positive ticks on positive dogs might have been infected by feeding. Similar findings have been reported in Poland (Zygner et al. 2008). We found no coinfection of Anaplasma and Borrelia in these ticks.

In ticks collected on wild animals, cows and horses in east Bohemia and Moravia, the DNA of A. phagocytophilum was detected in 9.8% of cases (Hulínská et al. 2004). The reported prevalences of infection of I. ricinus ticks with A. phagocytophilum were 1.1 1.4% in Switzerland (Liz et al., 2000, Wicki et al., 2000) and 3.2% in Slovenia (Petrovec et al., 1999). Our results are similar and correspond also to our result on ticks collected on dogs.

Bank voles and mice are known to host larval I. ricinus ticks. We found 17 adult ticks and several nymphs on 6 infected mice and 2 infected bank voles, but only three of the ticks hosted A. phagocytophilum. The amount of Anaplasma and Borrelia DNA in ticks depends on many factors: the tick’s nutrition status, the stage of the tick, the time of year, the geographical region, and the type of hosts.

We have described the prevalence of A. phagocytophilum and B. burgdorferi s. l. infections in the most important reservoir hosts in the Czech Republic, wild rodents, and determined the genospecies of B. burgdorferi s. l. associated with these hosts.

Our findings (Hulínská et al. 2004) of 13.3% infection with A. phagocytophilum DNA in samples from bank voles C. glareolus and 15% for yellow-necked mice A. flavicollis are comparable with findings in Switzerland (Liz et al., 2000) and in
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the UK (Bown et al., 2003). We found that the percentage of *Anaplasma* positive animals was higher in mice (15%) than in bank voles (13.3%).

Many species have been reported to serve as competent reservoirs of *B. burgdorferi* s.l. in Europe, in particular rodents of the genera *Clethrionomys* and *Apodemus* (Matuschka et al. 1992, Hu et al. 1997, Humair et al. 1999, Hanincová et al. 2003). We found *Borrelia* infection to be more common in *C. glareolus* than in *Apodemus* spp. (Kybicová et al. 2008, Zore et al. 1999). The most frequently captured rodent species were *A. flavicollis* and *C. glareolus*, the most abundant sylvan rodents in the Czech Republic and neighboring states (Hanincová et al. 2003). *B. afzelii*, *B. burgdorferi* s.s. and *B. garinii* were detected. To the best of our knowledge, this is the first report of the presence of *B. burgdorferi* s.s. in rodents in the Czech Republic. In our data, the majority of infections were caused by *B. afzelii* (Humair et al. 1995, Hu et al. 1997, Humair et al. 1998, Humair et al. 1999, Hanincová et al. 2003). A possible explanation is a reduced and shorter-lived infectivity of *B. burgdorferi* s.s. in comparison with *B. afzelii* which is better adapted to rodent hosts (Richter et al. 2004). However, *B. burgdorferi* s.s. was detected in small rodents in the UK, Poland, Ireland and Switzerland (Kurtenbach et al. 1998b, Humair et al. 1999, Gray et al. 2000, Michalik et al. 2005). *B. burgdorferi* s.s. seems to be less specialized and may be maintained both by avian and rodent hosts (Kurtenbach et al. 1998a). We have demonstrated one case of coinfection with *B. afzelii* and *B. burgdorferi* s.s. in *A. sylvaticus*, similarly as in Zore et al. 1999.

The presence of *Borrelia* was previously studied from ear biopsies and internal organs of small mammals (spleen, heart, liver, and urinary bladder) (Humair et al. 1993a, Zore et al. 1999, Hanincová et al. 2003, Christova et al. 2005). As ear biopsies may not reveal the full diversity of the infection, (Richter et al. 1999, Hanincová et al. 2003), we have used muscle samples, observing an infection rate of 16.4% (Kurtenbach 1998b, Christova et al. 2005). Seropositivity of wild rodents indicates previous exposure to infected ticks (Štefančíková et al. 2004). The prevalence of anti-*Borrelia* antibodies was higher in *Apodemus* spp. than in *C. glareolus* (Aeschlimann et al. 1986, Vostál and Žákovská 2003 and Štefančíková et al. 2004). The difference may be a result of either higher infestation of *Apodemus* spp. than *C. glareolus* by *Ixodes ricinus* ticks (Hanincová et al. 2003, Michalik et al. 2005) or by interspecies variability of the immune response (Kurtenbach et al. 1998a).

We have also investigated the occurrence of *A. phagocytophilum* in possible reservoir hosts in the Czech Republic, wild animals such as roe deer, fallow deer, red deer, mouflon sheep, European hare, red fox and wild boar. We have found the DNA of *A. phagocytophilum* in a higher percentage of cases in members of the deer family and hares (12.5–13.3%), as compared to foxes and boars (4% and
Similarly, in Spain, roe deer was infected in 18% of cases (de la Fuente et al. 2008). In Poland, roe deer were infected in 31.94% of cases (Adamska and Skotarczak 2007). In Slovakia, the deer family was infected in about 50% of cases and none of the wild boars was PCR positive (Stefanidesová et al. 2007). In Slovenia, somewhat higher percentage of *Anaplasma* positive cases in deer, 86% (Petrovec et al. 2002), red foxes *V. vulpes* and wild boar *Sus scrofa* were reported (Petrovec et al. 2003).

Besides vectors and reservoirs, we have been also interested in the occurrence of borreliosis and anaplasmosis in **hosts — domestic animals**, namely dogs, cows, and horses.

We have detected the DNA of *A. phagocytophilum* in 5% and 5.45% of cases, in horses and cows, respectively (Hulinská et al. 2004). Similarly results we obtained for *Anaplasma* in dogs (Kybicová et al. 2009). In Italy, horses were reported to be infected by *Anaplasma* in 8.14% of cases (Passamonti et al. 2010).

We have found the DNA of *A. phagocytophilum* in 3.4% (10 out of 296) of dogs (Kybicová et al. 2009). Similar PCR positivity rates, i.e. 6.3%, 5.5%, and 9.5% have been reported in Germany (Jensen et al. 2007), Italy (Torina et al. 2006), and USA (Beall et al. 2008), respectively. One out of our 155 healthy dogs was PCR positive for *A. phagocytophilum* which is a similar proportion to the study of Beall et al. 2008 in the USA (7 out of 222 healthy dogs). Morulae in peripheral blood granulocytes were observed in only two of the 10 PCR positive dogs, which correspond to the findings of Jensen et al. 2007 (2/6). The inclusion bodies produced by *A. phagocytophilum* appear in granulocytes and can be observed by microscopy only at the peak of the acute infection, which usually lasts only a few days (Engvall et al. 2002).

We detected DNA of *B. burgdorferi* s. l. in peripheral blood of only one PCR positive dog. This low yield can be explained by the transient nature of the presence of the spirochetes in the blood (Straubinger et al. 1997, Chang et al. 2001). Using PCR, borrelial DNA can only be detected in blood in the early stage of infection (Skotarczak et Wodecka 2003). Once the microorganism is disseminated in the body, a variety of organs can be affected, especially the skin, joints, heart and central and peripheral nervous systems (Straubinger et al. 1997).

The seropositivity rates in the examined dogs indicate natural exposure to *A. phagocytophilum* and *B. burgdorferi* s. l. We found a 25.9% positivity of IgG antibodies against *A. phagocytophilum*. For comparison, the reported canine seroprevalence rates were 17.7% for granulocytic *Ehrlichia* in Sweden (Egenvall et al. 2000a) and 7.5% for *A. phagocytophilum* in Switzerland (Pusterla et al. 1998). In Germany, antibodies to *A. phagocytophilum* were found in 43.2% of examined dogs (Jensen et al. 2007), in Israel in 9% (Levi et al. 2006), and in the USA, the canine seroprevalence rates were between 9.4% (Magnarelli et al. 1997) and 29%
The canine seroprevalence was reported to be 11.5% and 15.5% in Spain (Solano-Gallego et al. 2006, Amusategui et al. 2008) and 34.4% in Italy (Torina et al. 2006). All studies used IFA, except Beall et al. 2008, who used ELISA. The variability of reported seroprevalences may have resulted from the use of different selection criteria for examined dogs. We found no difference in seropositivity between symptomatic (27.5%) and asymptomatic (24.5%) dogs. Similar findings have been reported in Germany and USA (Jensen et al. 2007, Beall et al. 2008). The seropositivity in dogs without clinical signs can be explained by the persistence of antibodies as observed in chronically infected animals (Egenvall et al. 2000b). Seropositivity in many healthy dogs also indicates that antibodies to *A. phagocytophilum* in dogs can persist (Madigan et al. 1990, Magnarelli et al. 1997, Engvall et al. 2002).

We found 2.4% IgM and 10.3% IgG canine seropositivity rates for *B. burgdorferi* s. l. Serological detection of *B. burgdorferi* s. l. in dogs in the Czech Republic performed in 2006 (Pejchalová et al. 2006) showed a seroprevalence rate of 6.5%. The reported figures in other countries vary widely from 0.6% and 6.9% in Spain (Solano-Gallego et al. 2006, Amusategui et al. 2008*), 3.9% in Sweden (Egenvall et al. 2000a*), and 1.85% in Canada (Gary et al. 2006) to 11% in the USA (Beall et al. 2008), approximately 20% in France, the United Kingdom and Denmark (Doby et al. 1988*, May et al. 1991, Hansen and Dietz 1997*), 40.2% in Poland (Skotarczak et al. 2005) and 50% in Slovakia (Štefančíková et al. 1998). Most of the studies were based on ELISA, except for those marked by an asterisk (*), which were based on IFA. As mentioned above for *A. phagocytophilum*, the differences may have resulted from different dog selection criteria. Differences in canine seroprevalence rates for tick-borne diseases can also arise from variability in tick densities or proportion of infected ticks.

We also describe three cases of dogs naturally infected with borreliosis (Schánilec et al. 2010). All presented cases showed clinical and diagnostic features that strongly indicated borreliial infection. In all dogs, the clinical signs started between April and July. This corresponds with seasonal activities of ticks and dogs (Hovius et al. 1999a, Hovius et al. 1999b, Steere 2001, Bhide et al. 2004, Pejchalová et al. 2006, Kybicová et al. 2009). Clinical illness in infected dogs occurs 2 to 6 months after tick exposure.

Borreliosis is diagnosed based on the presence or absence of the following factors: 1) typical clinical symptoms; 2) exclusion of differential diagnosis; 3) reaction to antibiotics; 4) contact with a tick or living in an endemic area; 5) the presence of antibodies in the blood serum. The last criterion is often used as a diagnostic indication. However, seropositivity determined from a single sample cannot differentiate between past and present exposure, and should not on its own constitute a basis for diagnosis of an active infection (Egenvall et al. 2000a, Bhide
ELISA is considered to be sufficiently specific to be used for determining the dynamics of the antibody response to *B. burgdorferi s. l.*; it can detect an early stage of the illness (Hovius et al. 1999a). A correct timing of serologic examinations is important in order to determine whether active or past infection is responsible for the seropositivity. Early serodiagnostic results are usually negative because the immune response to borreliae develops gradually. Antiborrelial antibodies IgM are produced one to two weeks after the infection (Hovius et al. 1999a), correlate with the onset of the clinical illness, and remain elevated for two months (Greene and Straubinger 2006). IgG–ELISA positive titres develop within 4 to 6 weeks (Appel et al. 1993), culminate at 3 months and last 1 to 2 years after exposure (Straubinger et al. 2000, Goossens et al. 2001). Simultaneous measurements of IgG and IgM and paired-sample titres are recommended for detection of borrelial infection. False-negative antibody tests results are rare (Greene and Straubinger 2006). Clinical syndromes together with PCR and serial serology indicate a very likely borrelia induced disease. In all three of our dog case studies (Schānilec et al. 2010), the immune response and PCR findings indicated an early disseminated stage of a borrelial infection.

In Case 2 (Schānilec et al. 2010), neurological signs were reported but the origin of the seizures remained unclear. They could have been caused by nephro- or encephalopathy, because of azotemia or CNS borrelial infection cause irritation (Azuma et al. 1993, Azuma et al. 1994). The symptoms could have been caused by silent borrelial CNS infection with a transient relapse. Progressive renal failure, moderate to severe azotemia and significant proteinuria were detected along with IgM/IgG shift (Grauer et al. 1988, Greene and Straubinger 2006, Gerber et al. 2007). A possible increased sensitivity of labradors and golden retrievers to renal involvement has been described in the literature.

The ECG changes and post-mortem findings in Cases 1 and 3 make us think that the incidence of cardiac manifestations of borreliosis in dogs could be higher than previously thought. The observed cardiovascular syndromes include a presence of arrhythmia (variable degrees of atrioventricular block, supraventricular and ventricular arrhythmia) (Levy and Duray 1988, Nalmas et al. 2007), myocarditis, pericarditis, dilated cardiomyopathy and coronary artery disease (Gasser et al. 1999). The overall prognosis of Lyme carditis is good, although recovery may be delayed and later on the effects of inflammation and toxins can cause heart failure (Nagi et al. 1996, Gasser et al. 1999, Cepelová 2008). The chronic diffuse granulomatous and necrotic myocarditis found in Case 1 suggests the cystic form of the disease (Giudice et al. 2003).

A complete blood count (CBC) revealed persistent mild hypochromic anaemia in two cases (Case 1, 2) and borderline anaemia in the Case 3. Anaemia can appear...
in the acute stage of bacteraemia or inflammation because of bone marrow suppression or due to direct penetration of *B. burgdorferi* s. l. to bone marrow. The moderate panhypoproteinemia in the Case 1 and elevated alanine aminotransferase (ALT) (Cases 1, 2) can be a nonspecific effect of the systemic inflammation. In one case acute hepatitis was verified by necropsy.

We suggest that supportive complementary nursing care and cage rest of the dog patients are among the mainstays of the complex therapy.

We have used molecular methods for detection of these two tick-borne bacteria, *B. burgdorferi* s. l. and *A. phagocytophilum*. We tested different primer sets targeting 16S rDNA, *recA* gene for rapid detection in real-time PCR. We used RFLP analysis targeting 5S-23S intergenic spacer to differentiate *Borrelia* genospecies. To analyze the phylogenetic relationships between variants of *A. phagocytophilum*, we sequenced the PCR products of the 16S rDNA from our samples and created a phylogenetic tree.

Real-time PCR (RT-PCR) analysis of the *recA* gene (Fraser et al. 1997) is a rapid method for detection of *B. burgdorferi* s. l. with similar sensitivity as nested PCR (Pietila et al. 2000, Wang et al. 2003). We found the concordance between real-time PCR and RFLP analysis to be 97.6% (Kybicová et al. 2008). The melting curve analysis permitted to differentiate *B. garinii* from *B. afzelii* and *B. burgdorferi* s. s., but not to distinguish between *B. afzelii* and *B. burgdorferi* s. s., since the melting temperature difference can be as small as 1°C (Mommert et al. 2001, Wang et al. 2003, Casati et al. 2004). The RFLP method can confirm the real-time PCR results and is also able to distinguish between *B. afzelii* and *B. burgdorferi* s. s. (Derdáková et al. 2003).

Our results (Hulinská et al. 2004) by RT-PCR are comparable with previous examination of the HGA agent made with conventional PCR (Hulinska et al., 2002) and with observations in Europe (Liz et al., 2000) and in the United States (Belongia et al., 1997), where HGA is endemic. The sensitivity of detection of specific *Anaplasma* DNA in samples of infected animals using the six different RT-PCR methods was similar. We found that in naturally infected animals, there was generally less detection with the TaqMan Ep80 probe then when using the primers alone, contrary to the results on animals infected purposefully (Pusterla et al., 1999b). Differences in the melting curve analysis between HGA and variants of *A. phagocytophilum* in different RT-PCR assays were so small that they could not be used for distinguishing these variants, similar to *Borrelia* genospecies. However, our results indicate that the fluorescence melting curve analysis of PCR products can be used for a rapid detection of these agents (Wicki et al, 2000).

The direct sequence analysis of the 16S rDNA can be used for the detection of closely related *Anaplasma* species in humans and in animals (Chen et al., 1994, Liz...
et al., 2000). Sequence comparison of the 16S rDNA gene is recognized as one of the most powerful method for determining the phylogenetic relationships of bacteria as showed by Wen et al., 2002. Our findings (Hulínská et al. 2004) confirm that wild animals like deer and mice were infected with a similar variant of A. phagocytophilum as were ticks, cows and horses (Barlough et al., 1996). In the nested RT-PCR reaction the ECC-ECB primer pair produced a 520bp amplicon and the GER3-GER4 primer pair produced a 150 bp amplicon, containing the species-specific signature sequences. The choice of these amplicons for direct sequencing allowed differentiating between the bacterial variants. The phylogenetic tree that we constructed classifies the bacteria obtained from wild and domestic animals and tick as being closely related to the A. phagocytophilum genogroup.

Some authors (Petrovec et al., 1999, Liz et al., 2000, Wen et al., 2002) showed a high degree of similarity of partial 16S rDNA gene sequences of granulocytic A. phagocytophilum from ticks, rodents and deer with the HGA agent isolated from humans. Others (Massung et al., 2002, Baumgarten et al., 1999) found four variants differing in two nucleotides from the HGA or E.equi. They reported that more than one strain of A. phagocytophilum exists both in the United States, and in Europe. We found differences between sequences of A. phagocytophilum DNA extracted from a deer, boar, red fox, bank vole, tick and two horses in only 2–6 nucleotides.

In summary, our findings suggest that the exposure to B. burgdorferi s. l. and A. phagocytophilum is common in vectors, reservoirs and hosts in the Czech Republic. In vectors (ticks), we detected similar prevalence of both bacteria.

We also found a similar level of prevalence of Borrelia and Anaplasma in reservoir hosts (rodents). The majority of infections were caused by B. afzelii, while the infection with B. burgdorferi s. s. was also quite frequent. Infection with B. burgdorferi s. l. was more common in bank voles than in wood or yellow-necked mice. The prevalence of anti-Borrelia antibodies was higher in wood or yellow-necked mice than in bank voles. We conclude that small wild rodents can serve as hosts for B. burgdorferi s. l. and A. phagocytophilum in the Czech Republic.

We determined the occurrence of A. phagocytophilum in possible reservoir hosts. A. phagocytophilum was in a higher percentage of cases in the deer family and hares, as compared to foxes and boars.

Finally, we detected similar prevalence of anaplasmosis in domestic animals, such as cows, horses and dogs. We demonstrated that symptomatic dogs had higher chance to be infected with A. phagocytophilum than asymptomatic dogs.
Chapter 8

Conclusions

• The prevalence of both *B. burgdorferi* s. l. and *A. phagocytophilum* in ticks collected on animals was 6.8% and 9%, respectively. The prevalence of *A. phagocytophilum* in ticks from wild and domestic animals was very similar.

• In rodents, both *B. burgdorferi* s. l. and *A. phagocytophilum* were found in 16.4% and 14.5% of cases, respectively. This corresponds to other results in Europe reported in the literature.

• In wild rodents, the majority of infections were caused by *B. afzelii*, while the infection with *B. burgdorferi* s. s. was also quite frequent. To our best knowledge, this is the first report of the presence of *B. burgdorferi* s. s. in rodents in the Czech Republic.

• Infection with *B. burgdorferi* s. l. was more common in bank voles than in wood or yellow-necked mice. However the prevalence of anti-*Borrelia* antibodies was higher in wood or yellow-necked mice than in bank voles.

• The *Anaplasma* positivity detected by PCR was higher in yellow-necked mice than in bank voles.

• The infection rate of the deer family (12.5–15%), which is a possible reservoir for *A. phagocytophilum*, was found to be much lower than reported elsewhere in the literature.

• The prevalence of *A. phagocytophilum* in all tested domestic hosts (cows, horses, and dogs) was similar, 3.4–5.5%.

• We found that the exposure to *A. phagocytophilum* is common in dogs in the Czech Republic.
Chapter 8: Conclusions

- Symptomatic dogs had a higher chance to be infected with *A. phagocytophilum* than asymptomatic dogs.

- On the other hand, no significant correlation was found between clinical signs of anaplasmosis or borreliosis and positive antibody titers to *A. phagocytophilum* or *B. burgdorferi* s. l., respectively.

- The onset of clinical signs of borreliosis in dogs (April–July) corresponds with seasonal activity of ticks. The dogs infected by borreliosis suffered from neurological problems, renal failure, cardiovascular syndromes, inflammation, and anemia.

- Two of three infected dogs died, despite of antibiotic treatment, probably because the medical followup was prematurely terminated.

- Normally, borreliosis is associated with fever and orthopedic problems. However, two of the dogs did not suffer from fever and no dogs suffered from orthopedic problems.

- Wild animals (foxes, deer, boars, hares and rodents), domestic animals (cows and horses), and ticks collected from them were infected with similar variants of the same *A. phagocytophilum* genotype. The similarity was confirmed by a phylogenetic tree constructed from direct sequence analysis of the 16 rDNA fragments.

- We have used real-time PCR to determine the presence of *B. burgdorferi* s. l. in rodent muscle tissue. It is a very fast method but cannot distinguish between *Borrelia* genospecies. For this purpose we have successfully used RFLP analysis.

- We successfully adapted six published standard detection methods using PCR for real-time PCR, obtaining an important improvement in speed. However, real-time PCR was not capable of distinguishing the *A. phagocytophilum* variants.
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