Borrelia burgdorferi sensu lato and Anaplasma phagocytophilum in the Czech Republic

Summary of a submitted PhD thesis

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Summary

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.

V této práci jsou rovněž popsány molekulární a sérologické metody pro detekci obou patogenních bakterií, zahrnující konvenční PCR, nested PCR, real-time PCR s DNA kvantifikaci a analýzou teploty tání, RFLP analýzu 5S-23S mezigenového
mezerníku a přímou sekvenaci 16S rDNA. Real-time PCR je velmi rychlá metoda, která ale nedokáže rozlišit mezi genodruhy Borrelia ani mezi variantami A. phagocytophilum.

Práce popisuje i klinické příznaky a diagnostické nálezy u tří případů borreliové infekce psů. Ukazujeme, že je potřeba uvažovat o borreliové infekci nejen pokud jsou přítomny horečky nebo ortopedické potíže, ale i v mnoha jiných případech.
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.

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*
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 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.

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.
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%.

5 Summary of the results

Prevalence of B. burgdorferi s. l. and A. phagocytophilum detected by molecular (PCR) and serological (ELISA and IFA) methods
is shown in Table 1.
Table 1: Summary of detected prevalences. *B. b.* and *A. p.* stand for *Borrelia burgorferi* sensu lato and *Anaplasma phagocytophylum*, respectively.

<table>
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<th>PCR</th>
<th>Serum</th>
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<td><em>B. b.</em></td>
<td><em>A. p.</em></td>
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<tr>
<td>Vectors</td>
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<td>ticks from rodents</td>
<td>6.8%</td>
<td>9.8%</td>
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<td></td>
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<td></td>
<td>ticks from dogs</td>
<td>8.5%</td>
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<td>Reservoirs</td>
<td></td>
<td></td>
<td>bank voles</td>
<td>25.3%</td>
<td>13.3%</td>
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<td></td>
<td></td>
<td>mice</td>
<td>8.1%</td>
<td>15.0%</td>
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<td>Candidate reservoirs</td>
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<td>deer family</td>
<td>12.9%</td>
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<td>hares</td>
<td>12.5%</td>
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<td></td>
<td>foxes</td>
<td>4.0%</td>
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<td></td>
<td></td>
<td></td>
<td>boars</td>
<td>4.4%</td>
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<td>Domestic hosts</td>
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<td></td>
<td>cows</td>
<td>5.5%</td>
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<td></td>
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<td>horses</td>
<td>5.0%</td>
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<td>dogs</td>
<td>0.3%</td>
<td>3.4%</td>
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<td>IgM 2.4% IgG 10.3%</td>
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<td>IgG 25.9%</td>
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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 (Hulinská 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 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 4.35%) (Hulínská et al. 2004). 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 (Hulínská 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. 1. 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% (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 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— 15 —
1997*), 40.2% in Poland (Skotarczak et al. 2005) and 50% in Slovakia (Šťefančíková 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 et al. 2004, Skotarczak et al. 2005).

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 (Schánilec et al. 2010), the immune response and PCR findings indicated an early disseminated stage of a borrelial infection.

In Case 2 (Schánilec 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.
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 (Hulínská 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 than when using the primers alone, contrary to the results on animals infected purposely (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. a *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.
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.

- 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.
Curriculum vitae

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2006 degree RNDr. (Rerum Naturalium Doctoris) in biology from Faculty of Sciences, Charles University, Prague

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