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Detection of antibodies to *Borrelia burgdorferi* s.l. in wild small mammals and sensitivity of PCR and cultivation

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ABSTRACT

The aim of this study was to determine the seroprevalence of antibodies to *B. burgdorferi* s.l. in wild small mammals in the Czech Republic and compare sensitivity of PCR and cultivaton. Wild small mammals (n = 691) were trapped in years 2010–2014 in three localities of the Czech Republic. Heart rinses (n = 340) and sera (n = 351) were examined by modified indirect ELISA. Seventy animals were randomly selected for comparison of results of cultivation and PCR. Mean annual antiborelian positivity was 12% with statistical difference ($p < 0.05$) between Bank Vole (*Clethrionomys glareolus*) and other six animal species, while there was no statistical difference ($p > 0.05$) between rodentia and insectivora, gender and localities. The cultivation revealed one positive sample (1.4%), negative in both PCR and ELISA. Method PCR revealed seven positive samples (10%); two of them were simultaneously dubious in ELISA. Eleven animals, negative in cultivation and PCR, had antibodies in ELISA. Method of PCR compared to cultivation seems to be more sensitive for detection of *Borrelia*.

1. Introduction

Lyme borreliosis, also known as Lyme disease, is a wide-spread zoonosis and the most common vector-borne disease in Europe. It is caused by pathogenous spirochetes belonging to *Borrelia burgdorferi* sensu lato (*Bb*sl). It is an extracellular pathogen that uses several strategies to survive in an enzootic cycle involving a diverse range of hosts. Transmission of infection occurs through ticks and varies depending on the species of ticks and several other factors such as the vector's density and activity, abundance of reservoir animals and other blood hosts, land cover and human behavior and is influenced also by weather and climate (Jaenson and Lindgren, 2011). About 35 vertebrate species including wild small mammals are reservoir hosts of *B. burgdorferi* s.l. Rodents of genus *Apodemus* and *Clethrionomys* are typical reservoir hosts of *B. burgdorferi* s.l. in various enzootic areas in Europe (Kurtenbach et al., 1995).

The aim of this study was to determine the seroprevalence of antibodies to *B. burgdorferi* s.l. in wild small mammals in the Czech Republic and to compare the results of cultivation and PCR.

2. Materials and methods

2.1. Localities

The sampling was conducted in three localities (Poodří, Moravian Karst and Mohelno) at Moravian region of the Czech Republic. Poodří is localized in North Moravia (GPS: 49°44′N, 18°05′E) and trapping was carried out on 10 ha study plot in Bažantula forest, characterised by an oak Ficario-Ulmetum alnetosum association forests alternated with meadows. Moravian Karst is localized in South Moravia (GPS: 49°19′43.22″N; 16°43′23.52″E) and trapping was carried out in the surrounding of Skalní mlýn on 20 ha study plot. In this locality, there are beech forests with preserved species composition, complemented by oak and hornbeam forests. Mohelno is localized in South Moravia (GPS: 49°11′36.40″N, 16°16′21.63″E) and trapping was carried out along the river Oslava in the deep canyon valley in the reserve of Mohelno Serpentine Steppe on 4 ha study plot. Along the river, alluviums and ash trees have developed alongside the willows. In the hillside, then, oak and hornbeam in association Galio-Carpinetum dominate the river.

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Table 1
Prevalence of antiborrelian antibodies (IgG, IgM or both) tested by ELISA in wild small mammals at three localities in the Czech Republic.

Characteristic	Year of sampling					Total
	2010	2011	2012	2013	2014	
Rodentia						
Striped Field Mouse (*Apodemus agrarius*)	–	–	3/14 (21%)	0/18 (0%)	–	3/32 (9%)
Yellow-necked Field Mouse (*Apodemus flavicollis*)	6/59 (10%)	8/43 (19%)	20/127 (16%)	0/50 (0%)	13/174 (7%)	47/453 (10%)
Long-tailed Field Mouse (*Apodemus sylvaticus*)	–	1/5 (20%)	1/12 (8%)	0/2 (0%)	0/12 (0%)	2/31 (6%)
Bank Vole (*Clethrionomys glareolus*)		0/3 (0%)	27/105 (26%)	2/10 (20%)	1/33 (3%)	30/151 (20%)
Common Vole (*Microtus arvalis*)	–	–	–	0/6 (0%)	–	0/6 (0%)
Insectivore						
Eurasian Shrew (*Sorex araneus*)	–	0/1 (0%)	1/2 (50%)	0/8 (0%)	1/6 (17%)	2/17 (12%)
European Mole (*Talpa europea*)	–	–	–	0/1 (0%)	–	0/1 (0%)
Locality						
Poodří	6/59 (10%)	5/31 (16%)	14/43 (33%)	0/57 (0%)	0/36 (0%)	25/226 (11%)
Moravian Karst	–	4/21 (19%)	30/161 (19%)	2/38 (5%)	15/189 (8%)	51/409 (12%)
Mohelno	–	–	8/56 (14%)	–	–	8/56 (14%)
Total positivity (%)	6/59 (10%)	9/52 (17%)	52/260 (20%)	2/95 (2%)	15/225 (7%)	84/691 (12%)

2.2. Trapping of wild small mammals

Wild small mammals were trapped in the years 2010–2014 in spring and autumn by snapping traps and "life-hunt" traps. Traps were placed on ground in line at a distance of 7 m from each other. A total of 691 wild small mammals were trapped including 673 rodentia of four species and 18 insectivores of two species (Table 1). Blood obtained from the neck artery of anesthetized living individuals (n = 351) was used to obtain serum, that was stored at −18 °C. Annimals caught by snapping springs (n = 340) were dissected. The heart of each individual was put into 0.85% physiological solution for one day at temperature of 4 °C. After removing the heart, the remaining solution was centrifuged and the drained supernatant stored at −18 °C. In total, 691 samples of sera or heart rinses were collected.

2.3. Detection of antibodies to Borrelia burgdorferi s.l. by ELISA

Samples were examined by modified enzyme-linked immunosorbent assay (ELISA, TestLine, Prague, Czech Republic) used for the diagnosis of Lyme borreliosis in human medicine. The procedure was following: microplates were parallelly filled with 100 µl of respective antigen diluted in carbonate buffer at pH 9.6 (2 µg/ml) and incubated over night at 4 °C. Microplates were washed three times with phosphate buffer (pH 7.4) containing 0.05% Tween 20 (washing solution). Then 100 µl of sera (diluted at 1:100 in phosphate buffer with washing solution and 0.3% casein as binding solution) or heart-rinses (diluted at the same serum protein concentration) were inoculated into microplates and incubated at 37 °C for one hour. After a triple washing of the plates, 100 µl of anti-mouse IgG or IgM peroxidase conjugates (Sigma-Aldrich spol. s r.o., Prague, Czech Republic, diluted at 1:4000 and 1:3000, respectively with binding solution) were added per well. After one hour of incubation and a subsequent washing with washing solution, 100 µl of substrate solution (0.1 M citrate buffer pH 4.7–5.0 with 0.05% H_2O_2) with orthophenylene diamine were added per well. The reaction was stopped with one M H_2SO_4 after 20 min of incubation. The absorbance was measured at 492 nm on spectrophotometer (SLT RainBow, Schoeller instruments, s.r.o.). Mixture of ultrasonically disrupted whole cell antigens of *B. afzelii* BRZX27 MSLB 8065, *B. garinii* BRZX 23 MSLB 8064 and *B. burgdorferi* s.s. WSLB 8014/1 (Bioveta a.s., Ivanovice na Hané, Czech Republic) were used as antigen (Bbsl) in ELISA and for immunization of mouse to obtain positive control. Sera of wild mouse negative to Bbsl antigen were used as negative control. Sample was evaluated as positive, if it had IgM or IgG antibodies or both.

2.4. Comparison of sensitivity of cultivation and PCR

Sensitivity of cultivation and PCR was tested on seventy samples randomly selected from 260 small mammals collected in the year 2012. For cultivation, a piece (5 × 5 mm) of kidney, spleen and thigh muscle, was used. Tissues were separately placed into BSK-H medium (Sigma-Aldrich spol. s r.o) enriched with rifampicin and cultivated at 33–34 °C for 4–6 weeks and weekly checked by Dark field microscopy using darkfield condenser Olympus BX40 at magnification 400×. The same tissues samples were used to detect *B. burgdorferi* s.l. by PCR. DNA was isolated by GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich spol. s r.o.). The PCR based on the amplification of a specific sequence of a borrelial protein flagellin A was used (Picken et al., 1996). The PCR mixture contained 27 µl of mastermix containing primers (Mastermix kit Kit BBNHS *B. burgdorferi* s.l. nested hot start, GeneProof, Ltd., Brno, Czech Republic) and 3 µl of DNA. The PCR cycle consisted of 2 min at 37 °C followed with 10 min at 96 °C and 30 cycles of 96 °C for 10 s, 68 °C for 10 s, 72 °C for 40 s and extension 72 °C for 4 min, followed with next 45 cycles of 96 °C for 10 s, 54 °C for 10 s and 72 °C for 30 s. Gel electrophoresis was done on 2% agarose gel. The tissue with addition of *B. afzelii* (isolate BRZ30-X27) and the tissue of laboratory mouse negative for *B. afzelii* were used as positive and negative controls, respectively. The DNA isolation system and PCR was able to detect at least 1 to 10 borrelian cells in sample.

2.5. Statistical analysis

To determine whether the incidence of positive samples differ in animal species, localities and years, we used Fisher exact test (Chí-squared test) on the significance level of $p < 0.05$. One-way ANOVA test was used for multiple comparison procedures and LSD test for comparison of the homogenity of the sample. Student's *t*-test was used for analysis of difference in relation to gender. The cluster analysis was used for the statistical evaluation of ELISA positive, dubious and negative IgM and IgG samples. Data showed normal distribution based on the Shapiro–Wilk and Kolmogorov–Smirnov test.

3. Results

Mean annual antiborelian positivity for five years of study (2010–2014) was 12% without statistical difference ($p > 0.05$) between Rodentia (15%) and Insectivora (11%). The prevalence of antibodies in animal species ranged from 0% to 20% with statistical difference ($p < 0.05$) between Bank Vole (*Clethrionomys glareolus*) and other species (Table 1). The prevalence in individual years ranged from

2% to 20% with statistical difference ($p < 0.05$), while there was no statistical difference ($p > 0.05$) in seropositivity in relation to gender (10% for 337 males and 14% for 354 females) and localities (11%–14%).

By cultivation, we revealed one positive sample (1.4%), male Yellow-necked Field Mouse (*Apodemus Flavicollis*) from the locality Moravian Karst that was negative in both ELISA and PCR. Method PCR revealed seven positive samples (10%), four females and one male of Bank Vole, one male of Striped Field Mouse (*A. agrarius*) and one male of Yellow-necked Field Mouse. Two positive Bank Voles from Mohelno and Moravian Karst had simultaneously dubious result in ELISA. Eleven animals, negative in cultivation and PCR, had antibodies in ELISA.

4. Discussion

Blood serum is a standardized sample to determine the presence of specific IgG and IgM class antibodies, both in human (Tylewska-Wierzbanowska and Chmielewski, 2002), and rodent (Štefančíková et al., 2004). Heart rinse is rather a unique type of sample, however, it was chosen as a representative type of tissue, replacing the missing serum in dead rodents (due to residual blood in the myocardium) and also due to the frequent occurrence of some pathogens (*Borrelia burgdorferri* s. l.) in this tissue (Haddad and Nadelman, 2003; Grzesik et al., 2004). In our study, we detected antibodies to *B. burgdorferi* s.l. in 12% of wild small mammals that is lower compared to 17–19% prevalence in wild small mammals from Slovakia (Štefančíková et al., 2004, 2008) and 25–44% prevalence in previous studies from the Czech Republic, but in different localities (Vostal and Zakovska, 2002; Kybicová et al., 2008). Compared to mice, Bank Voles are supposed to have a stronger potential for transmission of *B. burgdorferi* s.l. to ticks (Radzijevskaja et al., 2013). We proved twice higher prevalence in *C. glareolus* (20%) compared to *Apodemus* spp. (10%), while it was reversed (11% and 20% for *C. glareolus* and *Apodemus* spp., respectively) in Slovakia (Štefančíková et al., 2004). In our study, we found statistical difference in prevalence in individual years. Based on the results of our long-term research, positivity for the presence of antibodies can change every year depending on natural conditions; the weather also affects the number of positive vectors and rodents.

Laboratory diagnosis of *B. burgdorferi* s.l. is based on serological examination with using sensitive ELISA. However, both false-negative and false-positive results do frequently occur. It can fail in the early phase of acute infection when a specific immune response has not occured yet or specific antibodies are detectable for months or years after complete recovery. *B. burgdorferi* s.l. is directly detectable by microscopic examination, cultivation and methods of molecular biology. Microscopy and cultivation are not available routinely and are less sensitive in cases of extracutaneous manifestations. Sensitivity of molecular methods varies depending on sample origin and the target. Negative PCR results do not exclude acute infection and positive results do not exclude presence of only DNA while bacteria are killed. That is why the results of different tests are often mismatched. In our study, we proved DNA of Bbsl by cultivation in one sample (1%) and by PCR in seven samples (10%). Method of PCR seems to be more sensitive for detectin of Bbsl. In Slovenia, *Borrelia* was proved by cultivation from lungs in 4% of animals and by cultivation from hearts in 22% of animals (Cerar et al., 2015); in Austria, *Borrelia* was proved by cultivation from bladder in 7% of animals (Khanakah et al., 2006). The differences could be due to, season and locality of trapping, different way of sampling and cultivations. In our previous research, rodent kidney, spleen and heart muscle were used for examination. Kybicová et al. (2008) showed that the rodent muscles exhibit a high degree of Bbsl. In case of PCR detection, similar result 6% was recorded in Bank Voles in France (Cosson et al., 2014), while it was higher in other European countries such as 39% in Slovenia (Cerar et al., 2015) and 15–24% in Austria (Khanakah et al., 2006; Schmidt et al., 2014).

Although ELISA and PCR analysis are widely used to assess the development of Lyme disease in infected animals, it is a question if the status of the infection is exactly evaluated. Nevertheless, this study shows that antiborrelian antibodies as well as DNA of *B. burgdorferi* s.l. are abundantly present in wild small mammals living in different localities of the Czech Republic.

Conflicts of interest

None.

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