West-to-east differences of Babesia canis canis prevalence in Dermacentor reticulatus ticks in Slovakia

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A B S T R A C T

Babesia canis canis is the most frequent causative agent of canine babesiosis in Central Europe, frequently causing severe disease. Recently, many new endemic foci of this disease have been reported from European countries. Growing incidence of canine babesiosis was recorded also in Slovakia during the last decade, from first cases in eastern Slovakia ten years ago to recent cases all over the south of the country. We have used nested PCR-RFLP method to study prevalence of B. c. canis in its natural tick vector Dermacentor reticulatus, collected at three geographically isolated lowland areas of southern Slovakia situated in the southeast, southwest, and west of Slovakia, respectively. The highest prevalence of B. c. canis was observed in D. reticulatus from eastern Slovakia (14.7%; n = 327), whereas the prevalence in southwest was significantly lower (2.3%; n = 1205). Notably, all 874 D. reticulatus ticks collected at Záhorská nižina lowland (W Slovakia) were B. c. canis-negative. Recorded differences in Babesia prevalence concurs well with the shift in incidence of clinical cases of canine babesiosis as observed by vet practitioners. Presented results revealed that eastern Slovakia represents an area of high risk of B. c. canis infection, whereas western areas of the country still remain Babesia canis-free.

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1. Introduction

Babesia canis, also known as large canine Babesia, can cause severe disease in infected dogs. Differences in B. canis antigen properties, vector specificity, geographic distribution and pathogenicity to dogs led to its splitting into three subspecies: (1) European B. c. canis transmitted by Dermacentor reticulatus, (2) South African B. c. rossi transmitted by Haemaphysalis ticks, and (3) widely distributed B. c. vogeli transmitted by ticks of the genus Rhipicephalus (Uilenberg et al., 1989; Zahler et al., 1998). Additionally, the fourth subspecies B. c. presentii having unknown vector has been detected in cats from Israel (Uilenberg et al., 1989; Carret et al., 1999; Baneth et al., 2004).

The most frequent causative agent of canine babesiosis in Central Europe is B. c. canis. This parasite activates antibody-mediated cytotoxic destruction of erythrocytes leading to anemia, haemoglobinemia, haemoglobinuria, trombocytopenia and, in case of massive infection, even to death because of multiple organ dysfunction syndrome (Lobetti, 2000; Zygner et al., 2007). During the last decade numerous new endemic foci of canine babesiosis were documented in European countries (i.a., Schwendewein, 1998; Zahler et al., 2000; Duh et al., 2004; Földvári et al., 2005; Matjila et al., 2005; Welc-Faleciak et al., 2009; Øines et al., 2010) probably in connection with an expanding range of its vector D. reticulatus (Gray et al., 2009). Range expansion of D. reticulatus was confirmed in Germany.
(Dautel et al., 2006), Poland (Zygner et al., 2009), Hungary (Srétet et al., 2005), and Slovakia (Bullová et al., 2009). The shifts in latitude and altitude reported for *D. reticulatus* range from Slovakia have been 200 km northward and 300 m higher, respectively, in last three decades (Nosek, 1972; Bullová et al., 2009).

Canine babesiosis was reported in Slovakia for the first time in 2000 from south-eastern part of the country (Chandoga et al., 2002), despite the fact that local veterinary practitioners probably noted its occurrence at least 2 years earlier (Kubelová, unpubl.). Afterwards, this disease was confirmed also in south-western Slovakia (Swan et al., 2001). Then, *Babesia c. canis* was diagnosed in *D. reticulatus* ticks in south-western Slovakia in 2002 (Duh et al., 2006). Subsequent reports on clinical cases of canine babesiosis in Slovakia imply its spreading north-westward to the Czech and Austrian border (Široký, unpubl.). Nevertheless, the Czech Republic is still considered to be *B. canis*-free country.

Based on the large number of *D. reticulatus* tick samples collected in three geographically separated areas of Slovakia, we set the following objectives for the paper: (1) to study presence and compare prevalence of *B. c. canis* in *D. reticulatus* ticks occurring in different areas of southern Slovakia; (2) to assess risk of *B. c. canis* infection for dogs freely moving through natural habitats at the south-east, south-west, and west of Slovakia; (3) to confirm east-to-west expansion of *B. c. canis* range as it is suspected under the Central European conditions.

2. Materials and methods

2.1. Collection of ticks

Adult ticks were collected by flagging (Sonenshine, 1993) from 37 localities distributed in three separated areas in eastern, south-western and western Slovakia, respectively, during September and October 2009 (Fig. 1, Table 1), the period corresponding to the second seasonal peak of *D. reticulatus* activity in studied area (Hornok, 2009). Sampling was carried out at places selected according to the expected habitat preferences of *D. reticulatus* published by Nosek (1972), namely at river basins, wet meadows, and shrub underflooded pastures. All sampled ticks were put into plastic vials, transported to laboratory for sexing and species determination (Nosek and Sixl, 1972), preserved in 96% pure ethanol and then stored at room temperature till DNA extraction.

2.2. DNA extraction

DNA from *D. reticulatus* was extracted by alkaline hydrolysis using modified method by Rijpkema et al. (1996). Ticks were removed from 96% ethanol, air dried, placed separately into test tubes containing 500 μl of 0.36M ammonium hydroxide, and left at room temperature for approximately 30 min. Then, the ticks in ammonium hydroxide solution were crushed by sterile single-use plastic pipette tips and boiled at 100 °C for 30 min. Thereafter, the tubes were opened and boiled at 100 °C for additional 20 min to remove remaining ammonia. Tubes with only ammonium hydroxide were included as negative controls into each isolation to detect possible cross-contamination. Concentration of dsDNA was measured using Micro-Volume Spectrophotometer ASP-3700 (Avans Biotechnology Corp., Taipei City, Taiwan) and Eppendorf BioPhotometer™ (Eppendorf A.G., Hamburg, Germany). Obtained lysates were stored at –20 °C.

2.3. PCR detection

Nested PCR detection of *B. canis* was carried out according to the modified protocol by Jefferies et al. (2007), employing external (BTF1, BTR1) and internal (BTF2, BTR2) primer sets. The primers amplify partial region of the 18S rRNA gene of *Babesia* and *Theileria* species with expected product lengths 930 bp and 800 bp, respectively. PCR was performed in a 25 μl volume containing 1 μl of DNA template, 0.625 Unit Taq Purple DNA Polymerase in PCR reaction buffer in volume 12.5 μl (Combi PPP Master Mix, Top-Bio s.r.o. Prague, Czech Republic), 10 pmol of each primer (Generi Biotech s.r.o., Hradec Králové, Czech Republic), and 9.5 μl of PCR water (Top-Bio s.r.o. Prague, Czech Republic). Amplification was performed using Life Express, XP Cycler (Bior, Hangzhou, China) and Bio-Rad MyCycler™ (Bio-Rad, Hercules, USA) thermal cyclers. An initial denaturation step at 94 °C for 10 min was followed by an initial activation sequence of steps as follows: 94 °C for 3 min, 63.6 °C for 1 min and 72 °C for 2 min. Then 45 cycles were run, each of 94 °C for 30 s, 63.6 °C for 30 s, and 72 °C for 30 s, followed by final extension step of 72 °C for 7 min. The same conditions were used for the second round of amplification, using 1 μl of PCR product of the first PCR as a template. The resulting PCR products were separated using 1.2% agarose gel and visualized by ethidium bromide under UV light.

2.4. RFLP analysis

PCR products (3 μl) from the second round of amplification were used for restriction digestion. All *Babesia*-positive samples were subjected to cleavage by 5 units of *HincII* and *BslI* at 47 °C and 2 units of *HinfI* at 37 °C overnight, respectively. Reaction mixture was prepared according to manufacturer’s protocol (New England Biolabs, Ipswitch, MA, USA) and the resulting fragments were separated using 1.2% agarose gel and visualized by ethidium bromide.

2.5. Sequencing of PCR products

To verify our results, representative part of positive PCR products was purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taipei, Taiwan) and sequenced using an ABI 3700 DNA Analyzer (Applied Biosystem, USA). Sequencing was provided by Macrogen, Korea. Obtained sequences were checked with Chromas v. 2.01 (Technelysium Pty Ltd) and compared to sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/). Results of PCR-RFLP were confirmed by sequencing of 30 randomly selected *B. c. canis*-positive samples. *B. c. canis* 18S rRNA partial sequences were comparable with highest similarity to
sequence published by Caccio et al. (2002); (accession number AY072926).

2.6. Data analysis

The prevalences found were mapped using ArcGIS software (McCoy and Johnston, 2001). We used 2-sample test for equality of proportions with continuity correction in R (R Development Core Team, 2010) to analyse variation in Babesia prevalence between sexes and areas.

3. Results

A total of 3493 ticks (3366 D. reticulatus and 127 Ixodes ricinus) from three different areas of Slovakia were collected. D. reticulatus were found at 30 localities whereas I. ricinus occurred at 23 localities. Subsequent nested PCR analysis carried out on selected 2406 D. reticulatus ticks representing all localities where this species was present revealed 76 Babesia-positive DNA samples (3.2%; n = 2406) (Fig. 1, Table 1). Exposing of these samples to single (HinfI) and double restriction digestion (HincII, BslI) displayed specific results for B. c. canis in all tested samples (Fig. 2).

Babesia prevalence in D. reticulatus females (3.5%; 45 out of 1276) was significantly higher ($\chi^2 = 5.64, df = 1, p = 0.017$) than the prevalence detected in males (1.9%; 21 out of 1130).

The highest number of positive samples (48) was diagnosed in eastern Slovakia at the vicinity of Michalovce, where B. c. canis was detected from ticks sampled at all...
five studied localities. The overall prevalence of *B. c. canis* in ticks originating from this area was 14.7% (*n* = 327), while prevalences in males and females were 12.9% (*n* = 147) and 16.1% (*n* = 180), respectively, with no difference between sexes detected (χ² = 0.43, df = 1, *p* = 0.51).

Significantly smaller prevalence of *B. c. canis* was detected in ticks sampled in south-western Slovakia near Gabčíkovo dam (2.3%, *n* = 1205; χ² = 80.7, df = 1, *p* < 0.001). Positive ticks originated from 5 out of 15 localities examined. Infection rates in males (2%; *n* = 591) and females (2.6%; *n* = 614) did not differ (χ² = 0.22, df = 1, *p* = 0.64). Concerning the third area representing western Slovakia, we did not detect any positive individual among the 874 diagnosed *D. reticulatus* ticks.

4. Discussion

Our results revealed considerable differences in *B. c. canis* prevalence among the three studied areas (14.7%,
The highest prevalence was detected in south-east Slovakia, the area where canine babesiosis was diagnosed for the first time in the country (Chandoga et al., 2002). Hence, we have clearly confirmed the presence of this B. c. canis natural focus. Significantly lower prevalence (2.3%) of B. c. canis in ticks was documented in south-western Slovakia at Gabčíkovo dam. There, our results are comparable to outcomes presented by Duh et al. (2006), who were the first recording B. c. canis in ticks from this region. They reported 1% prevalence of B. c. canis for ticks from the vicinity of Čuňovo village. Nevertheless, their sampling effort was considerably smaller. Duh et al. (2006) examined only 100 D. reticulatus with only one female being positive. Clinical cases of canine babesiosis are occasionally recorded in western Slovakia (Široký et al., unpubl.). However, we did not find any B. c. canis positive tick in this area. This could be caused by selection of sampled localities, which were concentrated in a relatively small area along the river Morava.

B. c. canis prevalence in the total sample was higher in female than male ticks at both Babesia-positive areas. Comparable results were reported by Zygnier et al. (2008), who described 9.5% positive males and 11.9% positive females. Our data suggest that the differences in prevalence between sexes are in fact small and their statistical significance can only be demonstrated in large samples.

There is paucity of studies concerning B. c. canis prevalence in D. reticulatus ticks. Rar et al. (2005a, b) reported 3.6% and 4.2% B. c. canis-positive D. reticulatus ticks in south-western and western Siberia, respectively. These values of prevalence coincide with our results. Significantly higher prevalence of B. c. canis (11% and 29%) was described by Polish and Hungarian researchers in ticks collected from dogs (Földvári et al., 2007; Zygnier et al., 2008). The prevalence reported from Poland (Zygnier et al., 2008) is comparable to south-eastern Slovakia, but the Hungarian ticks were infected at considerably higher rates (Földvári et al., 2007). Such data as those from south-eastern Slovakia, eastern Poland and Hungary indicate the presence of B. c. canis natural foci with high risk for dogs.

The decrease in prevalence values could also indicate trends in B. c. canis spreading in studied part of Central Europe from south-east (Hungarian lowland, southeastern Slovakia; Földvári et al., 2005, 2007; this study) to north-west (B. canis-free areas in westernmost Slovakia, Czech Republic, Germany, Austria; Dautel et al., 2006; Duh et al., 2006; Leschnik et al., 2008; this study). Endemic natural foci of canine babesiosis were known much earlier in Hungary than in Slovakia and Poland. Hence, higher prevalence in Hungary should not be surprising when compared to regions colonized more recently.

Despite the big amounts of restriction enzymes HincII and BsrII used under specific temperatures for considerably longer period than manufacturer recommends the distinguished part of non-digested PCR products was always detectable on agarose gel as additional band (Fig. 2). We consider important to pay appropriate attention to this problematic part of RFLP method as described by Jeffries et al. (2007). Low efficacy of HincII enzyme or exceeding amount of obtained amplified Babesia DNA were evaluated as probable reasons for this phenomenon. We have changed charge of enzymes in effort to optimize restriction digestion reaction two times, but unsuccessfully. We did not change amplification conditions in order to retain sensitivity and specificity rates of PCR. Minimal volume of 3 μl of PCR products was used in restriction digestion. Finally, the origin of these fragments was proved by sequencing.

Our study revealed that B. c. canis is quite common pathogen found over southern Slovakia. We give evidence that there is a west-to-east trend in prevalence suggesting its possible current spreading north-westwards in studied region. Comparing to high risk of B. c. canis infection of dogs in south-eastern Slovakia, western areas of the country remain safer in this sense. Extensive sampling is necessary to be carried out in western Slovakia to find exact limits of B. c. canis distribution range and to obtain data on threshold effects useful for predictive modeling of future range development.

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