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Molecular study of transovarial transmission of *Babesia canis* in the *Dermacentor reticulatus* tick

Ewa Julia Mierzejewska[1,A-D], Dorota Dwużnik[1,B], Anna Bajer[1,B,E-F]

[1] *Faculty of Biology, University of Warsaw, Poland*
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Abstract

The *Dermacentor reticulatus* tick is a main vector of *Babesia canis* in Europe. The risk of canine babesiosis is unpredictable, due to significant differences in the prevalence of *B. canis* between ticks originating from closely situated regions. This phenomenon may be explained by vertical transmission of the pathogen in a vector population. Thus, molecular techniques were applied to investigate the occurrence of transovarial transmission in *D. reticulatus* ticks.

DNA of *B. canis* was detected in 20.7% (6/29) of engorged female ticks collected from dogs, in every pool of eggs laid by positive females (100%, 6/6) and in larvae hatched from these eggs. In the pools of eggs collected from two positive females (2/6; 33.3%), no larvae hatched and no embryos were observed inside the eggs.

Conclusions. Transovarial transmission of *B. canis* can be an important mechanism supporting maintenance of the pathogen in the environment without the presence of a reservoir vertebrate host. However, the efficiency of transovarial transmission in the maintenance of *B. canis* in natural conditions requires further field research.

Key words

Dermacentor reticulatus, Babesia canis, transovarial transmission

INTRODUCTION

Babesia canis (Piana and Galli-Valerio, 1895) is a tick-borne haemoprotozoan pathogen causing canine babesiosis of dogs in Europe [1, 2, 3]. The main vector of these protozoa is the *Dermacentor reticulatus* tick (Fabricius, 1974) [3, 4]. Recent expansion of *D. reticulatus* in countries of Central Europe resulted in outbreaks of canine babesiosis in Netherlands [5], Switzerland [6] and Poland, among others [7]. However, as yet, in a newly-invaded territory the magnitude of canine babesiosis risk is impossible to predict. This results from significant differences in the prevalence of *B. canis* infection in ticks from different geographic regions, as demonstrated in a study in Poland [8]. Prevalence of *B. canis* infection in *D. reticulatus* in the Eastern and Western Polish populations was 5.42 and 0%, respectively. Significant differences in prevalence were also recorded between Northern (0.68%), Central (1.18%) and Southern (14.8%) areas in the expansion zone in Central Poland. In this study, the distances between particular locations were about 60–100 km. The reason of these differences is not known and may result from the availability of reservoir hosts, or the success of vertical transmission in the tick population. DNA of *B. canis* was found in several species of wild carnivores [9, 10], but none of them were recognized as a reservoir host for the parasite. Screening of clinically healthy dogs shows that only low numbers of individuals are asymptomatic carriers [11]. Thus, dogs do not seem to constitute a sufficient source of infection for ticks to support the maintenance of *B. canis* in certain localities.

Address for correspondence: Ewa Julia Mierzejewska, University of Warsaw, Faculty of Biology, ul. Miecznikowa 1, 02-096 Warszawa, Poland
e-mail: e.mierzejewska@biol.uw.edu.pl

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Transovarial transmission is an important mechanism for pathogen maintenance, allowing its survival even if a few generations of ticks never fed on a competent host [12]. Historically, *B. canis* was placed among the 'large' *Babesia* group on the basis of its morphology [2]. As a member of this group, *B. canis* was considered a parasite transmitted transovarially in ticks [2, 3, 13]. However, this phenomenon was never proved experimentally or in environmental conditions. Mehlhorn et al. [14] performed a microscopic study on *D. reticulatus* females experimentally fed on *B. canis*-positive dogs. Due to the results obtained, he proposed a full life cycle of *B. canis* with migration of its kinetes to tick ovaries and transovarial transmission. Mehlhorn et al. [14] underlined that the proposed scenario was hypothetical.

OBJECTIVES

To the best of our knowledge, transovarial transmission has never been studied in *D. reticulatus* using methods of molecular biology. Therefore the aim is to fill the gap in understanding the biology of *B. canis,* and apply molecular techniques to investigate the occurrence of transovarial transmission in *D. reticulatus* ticks.

MATERIALS AND METHOD

Collection of ticks, eggs and larvae. Fully engorged *D. reticulatus* females were collected from dogs during standard appointments in a veterinary clinic in Tłuszcz, Poland (N 52.426777, E 21.436375) in April and May 2017. *Dermacentor reticulatus* has been present in this territory for over two decades [11, 15] and in 2012–2014, the prevalence of *B. canis* in ticks from this area was 8.4% [8]. Engorged female ticks kept

separately in sterile vials were transferred to the laboratory. All vials were kept at the room temperature (20–22°C) in a moist chamber with a humidity of 95–100% for two weeks. Micro-environmental conditions were controlled daily by thermo-hygrometers (HOBO U23–001 Pro v2 Temperature/ RHData Logger, Onset Computer Corporation, USA). Females then oviposited eggs for three consecutive days. On the fourth day, half of the eggs from each female was gently separated using a sterile spatula and stored in the same conditions as described above to allow the larvae hatch. Females and the second half of the eggs were frozen at the temperature -20°C in separate sterile tubes. Pools of larvae from individual females were also frozen immediately after they had hatched.

DNA extraction. Tick females were dissected microscopically using sterile tools. The gut of the tick was removed carefully and all remnants of digested blood were thoroughly flushed out with PBS solution. The remaining internal organs were transferred to a sterile Eppendorf tube and homogenized using a plastic tissue grinder homogenizers. DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Germany) following the manufacturer's instructions.

Egg pools of weight 25mg and pools of 30 larvae originating from individual females infected with *B. canis* were subjected to DNA extraction, as described above. Efficiency of DNA extraction was checked by amplification of a fragment of tick 18S rDNA (600 bp) using primers 18S-F1/ 18S-R1 [16].

Amplification of Babesia canis DNA. Primers Bab GF2/ Bab GR2 were used to amplify a ~550 bp fragment of 18S rDNA in a one-step reaction [17]. Negative samples were then subjected to a nested-PCR reaction. In the first PCR, a ~1200 bp rDNA fragment was amplified with the outer primers CRYPTO R/ CRYPTO F [18]. In the nested PCR, primers Bab GF2/ Bab GR2 were used in two different approaches. In the first, the template was 0.5 µl of the first PCR product. In the second, 1.0 µl of 10x diluted post-reaction mixture was used as a template. All reaction conditions were set as previously described by the original authors. All negative controls were performed in the absence of template DNA. The positive control was the DNA of *Babesia microti* Kings College strain [19]. Amplicons were visualized with Midori Green stain (Nippon Genetics Europe GmbH) following electrophoresis in 2% agarose gels. Amplicons were purified and sequenced by a private company (Genomed SA, Poland). The resulting sequences were compared with sequences deposited in the GenBank database using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

A total of 29 fully-engorged *D. reticulatus* females successfully laid eggs. *Babesia canis* DNA was detected in six (20.7%) of the females. Prevalence of *B. canis* in this study was affected by specific conditions of the tick collection. All females were detached from dogs presented in a veterinary clinic due to alarming clinical symptoms, among others, typical for canine babesiosis.

Larvae hatched from all egg pools derived from uninfected females, but no larvae hatched from egg pools derived from two females infected with *B. canis* (2/6; 33.3%), and no embryos were observed inside the eggs.

DNA of *B. canis* was detected in all pools of eggs derived from infected females (6/6=100%), including two pools of unembronated eggs. All available pools of larvae from infected females were *B. canis*-positive (4/4=100%). Sequences obtained from all positive amplicons were identical and showed 99.6% similarity to nine sequences deposited in GenBank, including the one (KT272401) obtained from *D. reticulatus* during our previous study [8]. Therefore, we recorded successful transovarial transmission of *B. canis* in *D. reticulatus*. In recent studies focused on canine babesiosis outbreaks in the United Kingdom [20] and Switzerland [6], the autochthonous source of the disease was confirmed by detection of *B. canis*-positive ticks *D. reticulatus*. In both studies, the number of collected ticks was low. However, prevalence of *B. canis* was unexpectedly high: 82.4% (14/17) in the United Kingdom and 82.6% (19/23) in Switzerland. Given that larvae and nymphs of *D. reticulatus* are nidicolous and mainly feed on rodents (reservoir hosts of *B. microti* but not *B. canis*), it is likely that the ticks collected during these canine babesiosis outbreaks were progeny of a *B. canis*-positive female, and that the high infection of the offspring was a result of transovarial transmission.

Interestingly, impaired embryo development occurred only in eggs oviposited by *B. canis*-positive females. The phenomenon of reduced fitness and fertility of female ticks or decreased rate of survival of progeny were already documented in *D. andersoni* infected by *Rickettsia rickettsi* [21] and *Ixodes scapularis* heavily infected by *Borrelia burgdorferi* s.s. [22]. Reduced viability of *D. reticulatus* eggs could be a result of *B. canis* infection. The number of examined infected females in this study, however, is low. Eventual negative impact of *B. canis* infection on *D. reticulatus* reproduction needs further complex investigation. Studies focused on continuous transovarial transmission [12,23,24] demonstrated that pathogenic microorganisms cannot be maintained in perpetuity by the tick vector population. The intensity of pathogen infection and its virulence may be affected after repeated transovarial passages.

CONCLUSIONS

Transovarial transmission of *B. canis* may be an important mechanism supporting the maintenance of the pathogen in the environment, without the presence of a reservoir vertebrate host. Significant differences of *B. canis* prevalence in *D. reticulatus* ticks from close locations can be explained by this phenomenon. However, the efficiency of transovarial transmission in the maintenance of *B. canis* in natural conditions requires further field research.

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