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The role of juvenile *Dermacentor reticulatus* ticks as vectors of microorganisms and the problem of 'meal contamination'

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Abstract

Juvenile *Dermacentor reticulatus* ticks inhabit nests and burrows of their rodent hosts and cannot be collected from vegetation. To detect vertical transmission of *Babesia canis* in *D. reticulatus*, we studied larvae and nymphs collected from rodents. However, the molecular techniques used for detection of pathogen DNA are sensitive enough to detect not only pathogens vectored by ticks but also those taken up with current or previous blood meals ('meal contamination') or just present in the environment and on the tick or host surface ('environmental contaminations'). Thus, an additional aim of our study was to evaluate the extent of such contamination while studying feeding ticks collected from rodents. Juvenile *D. reticulatus* were collected from 140 rodents: 91 bank voles trapped in two forest sites in the Mazury Lake District and 49 rodents (*Apodemus* and *Microtus* spp.) from an open habitat near the town of Białobrzegi in Central Poland. Altogether 504 *D. reticulatus* ticks, comprising 266 individually evaluated nymphs and 238 larvae assigned to 50 larval pools, were studied for the presence of *Babesia, Bartonella* and *Rickettsia* spp. DNA. Statistical analyses were conducted to (1) evaluate the effect of rodent host factors (species, sex and age) on prevalence of infection in ticks, and (2) to compare the frequency of positive samples between groups of pathogen-positive and pathogen-negative rodent hosts. To complete the last aim, blood samples obtained from 49 rodents from Białobrzegi were studied for the presence of *Babesia* and *Bartonella* DNA. Infestation of rodent hosts with juvenile ticks ranged between 46 and 78%, with a mean abundance of 3.6 ticks/rodent for *D. reticulatus* and 4.8 ticks/rodent for *Ixodes ricinus*. The highest prevalence of PCR-positive *D. reticulatus* samples was obtained for *Rickettsia* spp. (28%) and *R. raoultii* was identified in 22 sequenced PCR products. *Babesia* DNA was detected in 20 (7.5%), including *B. microti* in 18 (6.8%) and *B. canis* in two (0.8%) of 266 *D. reticulatus* nymphs that were analyzed. *Babesia microti* DNA was also detected in four pools of *D. reticulatus* larvae (4/50 pools = 8%). The detection success of *B. microti* in *D. reticulatus* was associated with the species of the rodent hosts of the ticks (much higher for typical *B. microti*-host-species such as *Microtus* spp. than for *Apodemus* spp.) and host age (3×higher in ticks collected from adult hosts in comparison to juvenile ones). Moreover, the DNA of *B. microti* was detected in 68% of *D. reticulatus* nymphs collected from *B. microti*-positive rodents in comparison to only 1.6% of nymphs collected from *B. microti*-negative rodents. *Bartonella* DNA was

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detected in 18% of *D. reticulatus* tick samples (38% of larval pools, 14% of nymphs). Again, host factors played important roles for 'tick positivity'-the highest prevalence of positive ticks was on *Apodemus* spp., which are regarded as *Bartonella* reservoirs. *Bartonella* DNA was detected in 42% of nymphs and 57% of larval pools collected from *Bartonella*-positive rodents in comparison to 28% of nymphs and 11% of larvae collected from *Bartonella*-negative rodents. Vertical transmission of *B. canis* in *D. reticulatus* ticks was confirmed in the field. Additionally, we demonstrated that 'meal contamination' generates a confounding signal in molecular detection of pathogen DNA extracted from ticks collected from infected hosts and must be taken into account in evaluating the competence of tick species as vectors.

Keywords *Dermacentor reticulatus* · *Babesia canis* · *Babesia microti* · *Bartonella* · *Rickettsia raoultii* · Vertical transmission · Rodents

Introduction

Vertical transmission (transovarial or transstadial) is known to play a crucial role in the maintenance of tick-borne pathogens in tick populations. To confirm transovarial transmission, naïve (unfed) or questing larvae of certain tick species need to be examined for the presence of relevant pathogens. Similarly, for transstadial transmission questing and as yet unfed nymphs should be the focus of investigation. Collection of questing instars from vegetation and the environment is relatively easy for exophilic tick species, i.e. *Ixodes ricinus*, however cannot be accomplished for endophilic tick species. In the case of *Dermacentor reticulatus* juvenile ticks inhabit nests and burrows of their rodent hosts from which they do not emerge and hence cannot be collected from vegetation (Rubel et al. 2016).

The ornate dog tick, *D. reticulatus,* is widely regarded as the main vector of *Babesia canis* (Rar et al. 2005a; Mierzejewska et al. 2015a; Rubel et al. 2016). The recent spread of this tick species in many European countries (Matjila et al. 2005; Schaarschmidt et al. 2013; Földvári et al. 2016; Mierzejewska et al. 2016) has resulted in the contemporaneous spread of canine babesiosis and has raised questions about the possible zoonotic reservoir of *B. canis* and the possibility of vertical transmission of the piroplasm in the tick population. To investigate the possibility of vertical transmission of *B. canis* in *D. reticulatus* under natural conditions, we studied larvae and nymphs collected from rodents since free-ranging juveniles are not available on vegetation.

Ticks collected from hosts usually present a range of engorgement levels (Mierzejewska et al. 2015b), however even recently attached specimens which are still identical in morphological indices and weight with questing co-mates, should not be treated as totally naïve and unfed. Feeding ticks are in contact with a range of host tissue: blood, skin, connective tissue. Molecular techniques used for the detection of pathogen DNA in ticks are sensitive enough to detect pathogens present in host tissues in addition to these vectored or hosted by ticks (Egyed and Makrai 2014). Moreover, some studies have reported detection of host and pathogen DNA from previous blood meals, completed in earlier stages of tick life (Pichon et al. 2003; Wodecka and Skotarczak 2016). In summary, applying PCR-based sensitive techniques for the detection of pathogen DNA in feeding ticks may result in the detection of 'meal contamination' in cases of ticks feeding or having fed on infected hosts or in the detection of microorganisms present in the environment and contaminating tick or host surfaces. Thus, an additional aim of our study was to evaluate the extent of such contamination while studying feeding ticks collected from rodents, which are often naturally

infected with a range of vector-borne pathogens (Welc-Faleciak et al. 2008a, b; Paziewska et al. 2011; Bajer et al. 2014; Tolkacz et al. 2017).

To differentiate between 'meal contamination' and the presence of specific pathogens in ticks, we determined the infection status of rodent hosts with the corresponding pathogens and compared this with the detection of pathogen DNA in ticks from infected and non-infected hosts. Thus the aims of our study were: (1) to investigate the occurrence of vertical transmission of *B. canis* in *D. reticulatus* ticks under natural conditions; (2) to evaluate the vector role of juvenile *D. reticulatus* for other pathogens; (3) to evaluate the effect of rodent factors (host species, sex and age) on detection of pathogen DNA in tick samples; and (4) to compare the frequency of positive samples between groups of pathogen-positive and pathogen-negative rodents.

Materials and methods

Field study

Ticks were collected from rodents at four sites. Two sites, Urwitałt and Tałty, are located in the Mazury Lake District, in NE Poland. These two forest sites and the associated trapping procedures have been described in detail in our previous papers (Behnke et al. 2001, 2008a, b; Bajer et al. 2014). Ninety-one bank voles, *Myodes glareolus*, were trapped and inspected for ectoparasites from these two sites in August 2016 (Table 1).

Two additional sites, Białobrzegi (N 51.6587, E 20.9388) and Niewiadów (N 51.6237, E 19.9150), were selected in the expansion zone of *D. reticulatus* in the Mazovia region in Central Poland (Mierzejewska et al. 2016). These two sites comprised open habitats, fallow lands and abandoned meadows, and the rodent community at these sites consisted of typical open habitat species: several *Microtus* spp. voles and striped field mice *Apodemus agrarius*, with occasional visiting forest species (*A. flavicollis, A. sylvaticus, M. glareolus*) (Table 1). Urwitałt, Tałty and Białobrzegi are localized in areas endemic for *D. reticulatus*, as determined by the presence of adult ticks on vegetation (Mierzejewska et al. 2016). Niewiadów is located about 70 km to the West from Białobrzegi in an area historically free from *D. reticulatus* (Siuda 1993; Karbowiak 2009; Mierzejewska et al. 2016). This site was selected also to verify the actual range of *D. reticulatus* ticks in Poland.

Trappings of rodents was performed during the known period of activity of juvenile *D. reticulatus* in Poland (Karbowiak 2009). Rodents were trapped in June and August 2017 in Białobrzegi and Niewiadów, and in August 2016 in Urwitałt and Tałty (Table 1).

All procedures have been described in detail in our previous papers (Behnke et al. 2001, 2008a, b; Bajer et al. 2014). Briefly, rodents were live-trapped, transported to the laboratory in Urwitałt or processed at the trapping site, for trapping in the Mazury Lake District (Masuria) and in Mazovia, respectively. In Urwitałt rodents were inspected for ectoparasites after blood collection from the heart under terminal isoflurane anesthesia (Behnke et al. 2008a, b; Tołkacz et al. 2017). In Białobrzegi and Niewiadów rodents were inspected following non-terminal isoflurane anesthesia and released afterwards, as described previously (Tołkacz et al. 2017). Ectoparasites were collected into 70% ethanol. Blood smears were prepared from blood taken from the heart (Masuria) or tail tip (Mazovia). Additionally, blood samples were collected into 0.001 M EDTA for DNA extraction and molecular detection of pathogens in hosts.

Table 1 Origin of tick samples: number of *Dermacentor reticulatus* larvae and nymphs collected from rodents at four sites

Area	Region	Site	Habitat	Month and year	Host species and numbers	*D. reticulatus* larvae (n)	*D. reticulatus* larvae pools (n)	*D. reticulatus* nymphs	Total *D. reticulatus* (no. of tick samples including pools)
Endemic for *D. reticulatus*	Mazury Lake District, NE Poland	Urwitałt	Forest	August 2016	*Myodes glareolus* n=52	1	1	142	143 (143)
		Tałty			*M. glareolus* n=39	1	1	44	45 (45)
		Combined			**Total *M. glareolus* n=91**	**2**	**2**	**186**	**188 (188)**
	Mazovia, Central Poland	Białobrzegi	Fallow lands	June and August 2017	***Apodemus agrarius* n=21**	**39**	**10**	**28**	**67 (38)**
					Apodemus flavicollis n=11	74	15	1	75 (16)
					Apodemus sylvaticus n=2	28	3	0	28 (3)
					***Apodemus* spp. (*A. flavicollis*+*A. sylvaticus*) n=13**	**102**	**18**	**1**	**103 (19)**
					Microtus arvalis n=1	0	0	0	0
					Microtus agrestis n=3	44	7	0	44 (7)
					Microtus oeconomus n=8	17	6	50	67 (56)
					Microtus sp. (unident) n=3	34	7	1	35 (8)
					Total *Microtus* spp. n=15	**95**	**20**	**51**	**146 (71)**
					Total rodents n=49	**236**	**48**	**80**	**316 (128)**

Table 1 (continued)

Area	Region	Site	Habitat	Month and year	Host species and numbers	D. reticulatus larvae (n)	D. reticulatus larvae pools (n)	D. reticulatus nymphs	Total D. reticulatus (no. of tick samples including pools)
Non-endemic for *D. reticulatus*	Mazovia, Central Poland	Niewiadów	Fallow lands	June and August 2017	*Myodes glareolus* n=1	0	0	0	0
					Apodemus agrarius n=11	**0**	**0**	**0**	**0**
					Apodemus flavicollis n=3	0	0	0	0
					Microtus oeconomus n=19	0	0	0	0
					Total rodents n=34	**0**	**0**	**0**	**0**
Overall total					Rodents n=174	238	50	266	504 (316)
Endemic area					Rodents n=140	238	50	266	504 (316)
Non-endemic area					Rodents n=34	0	0	0	0

In bold the host species/genera used as separate groups in statistical analyses

Positive and negative controls were incorporated in each set of PCRs and selected PCR products obtained from tick samples and rodents were sequenced by a private company (Genomed, Warsaw, Poland).

Sequence analysis

DNA sequence alignments and analyses were conducted using MEGA v.7.0. Consensus sequences were compared with sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/).

Statistical analyses

Statistical analyses were conducted to: (1) evaluate the impact of host factors (rodent host species, sex and age) and study site on the detection of pathogen DNA in ticks collected from rodents, and (2) to associate the infection identified in tick samples with the infection status of the rodent hosts from which the ticks were collected.

For the first aim we analyzed the prevalence of pathogens (% PCR-positive samples) in *D. reticulatus* tick samples. The statistical approach adopted has been documented comprehensively in our earlier publications (Behnke et al. 2001, 2008a, b). For analysis of prevalence we used maximum likelihood techniques based on log linear analysis of contingency tables in the software package SPSS (v.21, SPSS, Chicago, IL, USA). Initially, full factorial models were fitted, incorporating as factors rodent hosts of the ticks HOST SPECIES (four groups as explained below), SEX (two levels, males and females), AGE (two levels: juveniles and adults, based on breeding status) and SITE (4 levels, the four study sites). Four host species/genera were established for statistical analyses grouping open habitat species (group 1: *A. agrarius*; group 2: *Microtus* spp. [*M. arvalis* + *M. agrestis* + *M. oeconomus* + unidentified *Microtus* spp.]) and forest species separately (group 3: *Apodemus* spp. [*A. flavicollis* + *A. sylvaticus*]; group 4: *M. glareolus*). Prevalence of pathogens in ticks was considered as a binary factor (PRESENCE/ABSENCE of pathogen DNA in ticks). These explanatory factors were fitted initially to all models that were evaluated. For each level of analysis in turn, beginning with the most complex model, involving all possible main effects and interactions, those combinations that did not contribute significantly to explaining variation in the data were eliminated in a stepwise fashion beginning with the highest level interaction (backward selection procedure). A minimum sufficient model was then obtained, for which the likelihood ratio of χ^2 was not significant, indicating that the model was sufficient in explaining the data (these values are given in the legends to the figures as relevant). The importance of each term (i.e. interactions involving infection) in the final model was assessed by the probability that its exclusion would alter the model significantly and these values relating to interactions that included PRESENCE/ABSENCE of pathogen DNA are given in the text.

For the second aim, additional analyses were performed, incorporating additional factor in the model: HOST INFECTION (PRESENCE/ABSENCE of pathogen in the rodent host of the tick), as determined by PCR. Two models were analyzed for *B. microti* and *Bartonella* spp.: HOST SPECIES × HOST INFECTION × TICK STADIUM × TICK INFECTION. In these analyses tick samples (n = 128) and rodents (n = 49) from Białobrzegi were used.

General linear models (GLMs in SPSS v.21) were used for comparison of mean tick abundance between sites.

Results

Tick infestation in rodents

A total of 174 rodents were examined for ticks, including 92 *M. glareolus*, 32 *A. agrarius*, 14 *A. flavicollis*, two *A. sylvaticus*, 27 *M. oeconomus*, three *M. agrestis*, one *M. arvalis* and three juvenile *Microtus* voles (*Microtus* spp.) (Table 1), which could not be identified to species level because of their very small body size (juvenile *M. agrestis* and *M. oeconomus* voles are morphologically almost identical).

Altogether 1393 feeding ticks were collected from these rodents at four sites, including 504 *D. reticulatus* (238 larvae and 266 nymphs) and 889 *I. ricinus* (852 larvae and 37 nymphs). *Ixodes ricinus* was the most common species found on rodents at all sites (Fig. 1a). Total prevalence of this species on rodents ranged between 69 and 91%, mainly due to the high infestation with *I. ricinus* larvae (Fig. 1a, NS). Mean abundance was also
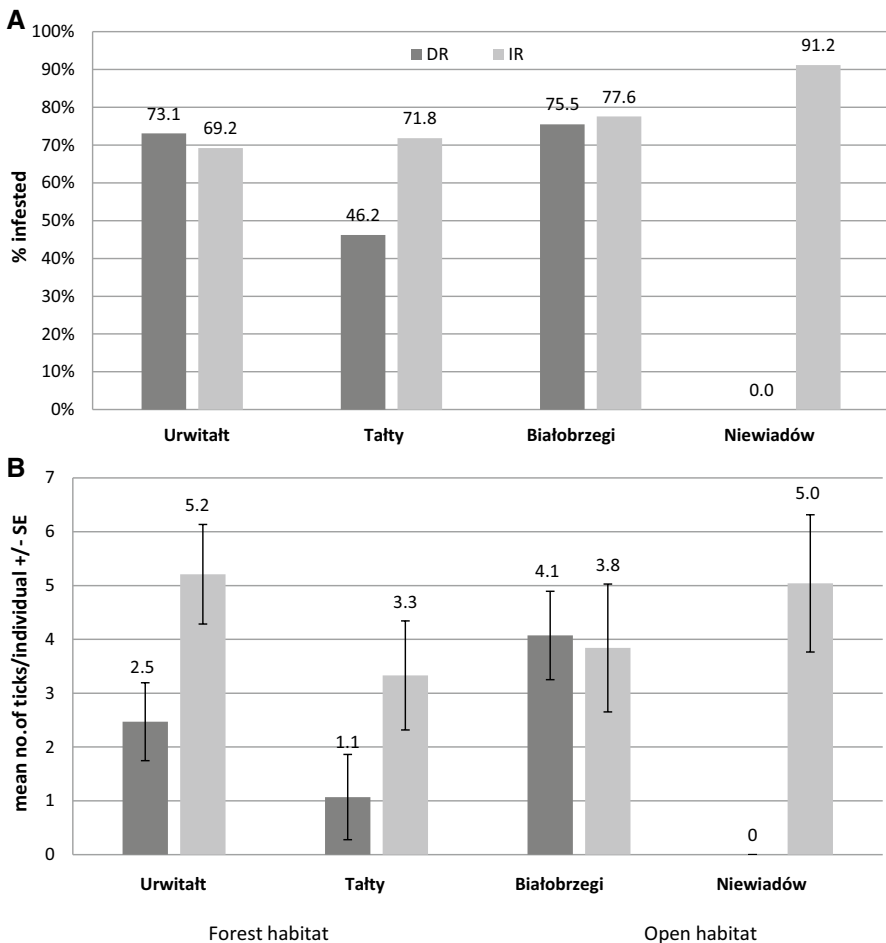

Fig. 1 Comparison of the prevalence of tick infestations (**a**) and abundance (**b**) on rodents from four trapping sites. *DR D. reticulatus*, *IR I. ricinus*

similar between sites (Fig. 1b, NS). Juvenile *D. reticulatus* ticks were found on several species of rodents (Table 1) at three sites; no ticks of this species were recorded on rodents from Niewiadów (Fig. 1a, b; *D. reticulatus* PRESENCE/ABSENCE × SITE: $\chi_3^2 = 71.4$, $P < 0.001$), supporting the hypothesis that this site is located outside the geographical range of *D. reticulatus*. Thus, rodents from this site were excluded from further analyses. The mean abundance of *D. reticulatus* ticks on rodents in tick endemic areas was 3.6 ticks/host, a value that is similar to the mean abundance of juvenile *I. ricinus* (4.8 ticks/hosts). Mean abundance of *D. reticulatus* was highest on rodents caught in the open habitat in Białobrzegi and lowest at the forest site in Tałty (Fig. 1b; main effect of SITE on tick abundance: $F_{3, 173} = 3.84$, $P = 0.011$).

Molecular detection of pathogen DNA in juvenile *Dermacentor reticulatus* and rodent hosts

The overall frequencies (% positive) of detection of specific pathogen DNA in larvae and nymphs of *D. reticulatus* are presented in Table 2. The frequency of detection of pathogen DNA in *D. reticulatus* originating from different host species and from the three trapping sites where this tick species occurred is presented in Table 3, and it is clearly apparent that some host species dominated in each case. To enable comparison of infection of ticks and their rodent hosts, selected positive samples from ticks and rodents (Table 4) were sequenced and analyzed. Among the detected pathogens, *Rickettsia* was the most common and the rarest was *Babesia* spp.; a relatively high % of *Bartonella*-positive samples were recorded among the pools of *D. reticulatus* larvae (Tables 2 and 3).

Babesia spp.

Altogether 42 PCR products of *Babesia* spp., including 24 samples from ticks (all positive; Table 2) and selected 18 samples from rodents (Table 4; 15 *M. oeconomus*, 2 *A. agrarius*, 1 *A. flavicollis*) were sequenced. Two *Babesia* species were identified: both *B. microti* and *B. canis* in ticks but only *B. microti* in rodents. All *B. microti* sequences from rodents and all but one sequence from ticks showed the closest similarity (99.6–100%) to *B. microti* IRU1 genotype (KC470048). Only one *B. microti* sequence obtained from a *D. reticulatus* nymph from *A. agrarius* was identical with *B. microti* IRU2 genotype (KC470049). Two sequences obtained from the nymphs of *D. reticulatus* (one collected from *M. glareolus* from Urwitałt,

Table 2 Detection of pathogen DNA in larvae and nymphs of *Dermacentor reticulatus* from endemic areas

Frequency (% positive) of pathogen DNA detection

	No. of examined tick samples	*R. raoultii* I/N	*Bartonella* spp. I/N	*B. microti* I/N	*B. canis* I/N
Larvae (pools)	50	21/50 42.0%	19/50 38.0%	4/50 8.0%	0/50 0.0%
Nymphs	266	67/266 25.2%	38/266 14.3%	18/266 6.8%	2/266 0.8%
Combined	316	88/316 27.9%	57/316 18.0%	22/316 7.0%	2/316 0.6%

I—number of positive tick samples; N—number of examined tick samples

Table 3 Frequency of DNA detection of selected pathogens in *Dermacentor reticulatus* tick samples originated from certain host species

Area	Region	Site	Month and year	Host species of examined D. reticulatus	Babesia canis I/N % positive	Babesia microti I/N % positive	Bartonella spp. I/N % positive	Rickettsia spp. I/N % positive
Endemic for D. reticulatus	Mazury Lake District, NE Poland	Urwitałt	August 2016	*Myodes glareolus*	1/144 0.7%	3/144 2.1%	12/144 8.3%	50/144 34.7%
		Tałty		*Myodes glareolus*	0/44 0%	3/44 6.8%	2/44 4.5%	11/44 25%
		Combined		Total *M. glareolus*	**1/188 0.5%**	6/188 3.2%	14/188 7.4%	61/188 32.4%
	Masovia, Central Poland	Białobrzegi	June and August 2017	*Apodemus agrarius*	0/38 0%	1/38 2.6%	13/38 34.2%	10/38 26.3%
				Apodemus flavicollis	0/16 0%	0/16 0%	9/16 56.3%	6/16 37.5%
				Apodemus sylvaticus	0/3 0%	0/3 0%	3/3 100%	2/3 66.7%
				Apodemus spp. (A. flavicollis + A. sylvaticus)	0/19 0%	0/19 0%	**12/19 63.2%**	8/19 42.1%
				Microtus agrestis	0/7 0%	3/7 42.6%	2/7 28.6%	5/7 71.4%
				Microtus oeconomus	**1/56 1.8%**	11/56 19.6%	**15/56 26.8%**	1/56 1.8%
				Microtus sp. (unident)	0/8 0%	1/8 12.5%	1/8 12.5%	3/8 37.5%
				Total *Microtus* spp.	**1/71 1.4%**	**15/71 21.1%**	18/71 25.4%	9/71 12.7%
				Total rodents	**1/128 0.8%**	16/128 12.5%	43/128 33.6%	27/128 21.1%
	Combined	Combined		Overall total rodents	**2/316 0.6%**	22/316 7%	57/316 18%	88/316 27.8%

In bold the host species/genera used as separate groups in statistical analyses

Tick samples combined individually examined nymphs and pools of larvae (details in Table 1)

one from *M. oeconomus* from Białobrzegi) were essentially identical (449/450 = 99.8% and 435/435 = 100%) to the known sequence of *B. canis* (KY021189). Thus, the total frequency of *Babesia* DNA detection in juvenile *D. reticulatus* was 7.6%, including 7% of tick samples positive for *B. microti* and two samples (0.6%) positive for *B. canis* (Table 2). *B. microti* DNA was detected in eight rodents from Białobrzegi (16.3%; Table 4). Host species had a significant effect on the prevalence of *B. microti* infection in rodents as reflected in the outcome of an analysis of presence/absence of *B. microti* in rodents from Białobrzegi (*B. microti* HOST INFECTION x HOST SPECIES: $\chi_3^2 = 19.5$, $P < 0.001$). The highest prevalence of *B. microti* was identified in *Microtus* spp. (Table 4).

Bartonella spp.

Bartonella spp. DNA was detected in 57 juvenile *D. reticulatus* (18%). Twenty-two PCR products were sequenced, including ten sequences from larval pools and 12 sequences from nymphs (Table 2). Three species/genotypes of *Bartonella* were identified; ten sequences displayed the highest similarity (99–100%) to *B. grahamii* (KC633098); nine sequences were most similar to *B. taylorii* (99–100%; MG839176) and three sequences were identical with the *B. rochalimae*-like isolate (MG839175). *Bartonella* DNA was detected in 38% of larval pools and in 14% of nymphs (Table 2).

Statistical analysis of *Bartonella* infections in ticks and rodents from Białobrzegi revealed a significant interaction between factors, including HOST SPECIES (*Bartonella* HOST INFECTION x HOST SPECIES x TICK STADIUM: $\chi_3^2 = 11.7$, $P = 0.008$). *Bartonella* DNA was detected in 22 rodents from Białobrzegi (45%; Table 4), with highest prevalence in mice, *Apodemus* spp. (84.6%) but these infections in rodents were not isolated and sequenced.

Rickettsia spp.

Rickettsia DNA was detected in 88 *D. reticulatus* tick samples (28%; Table 2). Twenty two PCR products were sequenced (nine from larval pools and 13 from nymphs). All sequences represented just one genotype, 100% identical with *R. raoultii* (KY474576). Rodent samples were not examined for *Rickettsia*.

Host factors affecting frequency of DNA detection in ticks

Rodent host species/genus (four groups as described in the "Materials and methods" section), host age and sex were fitted as factors influencing PRESENCE/ABSENCE of pathogen DNA in *D. reticulatus* ticks. The effect of these host factors on the frequency of pathogen detection in ticks is presented in Fig. 2a–c.

Host species/genus had a significant effect on the frequency of detection of three pathogens in *D. reticulatus* (Fig. 2a, Table 3). *B. microti* DNA was not detected in ticks collected from *Apodemus* spp. but the highest percentage of positive samples was recorded for ticks collected from *Microtus* spp. (*B. microti* PRESENCE/ABSENCE × HOST SPECIES: $\chi_3^2 = 17.0$, $P = 0.001$). The lowest frequency of samples positive for *Bartonella* spp. was recorded in ticks collected from *M. glareolus* and the highest in ticks collected from *Apodemus* spp. (*Bartonella* PRESENCE/ABSENCE × HOST SPECIES: $\chi_3^2 = 37.6$, $P < 0.001$). The lowest frequency of samples positive for *Rickettsia* was recorded in ticks

Table 4 Detection of pathogen DNA in rodent samples from Białobrzegi and Niewiadów

Area	Region	Site	Habitat	Month and year	Host species and numbers	Babesia microti I/N % infected	Bartonella spp. I/N % infected
Endemic for *D. reticulatus*	Mazury Lake District, NE Poland	Urwitałt	Forest	August 2016	*Myodes glareolus* n=52	ND	ND
		Tałty			*M. glareolus* n=39	ND	ND
		Combined			Total *M. glareolus* n=91	ND	ND
	Mazovia, Central Poland	Białobrzegi	Fallow lands	June and August 2017	***Apodemus agrarius* n=21**	**2/21** **9.5%**	**4/21** **19%**
					Apodemus flavicollis n=11	2/11 18.2%	10/11 91%
					Apodemus sylvaticus n=2	0/2 0%	1/2 50%
					***Apodemus* spp.** **(*A. flavicollis* + *A. sylvaticus*)** **n=13**	**2/13** **15.4%**	**11/13** **84.6%**
					Microtus arvalis n=1	1/1 100%	0/1 0%
					Microtus agrestis n=3	1/3 33%	2/3 66.7%
					Microtus oeconomus n=8	2/8 25%	3/8 37.5%
					Microtus sp. (unident) n=3	0/3 0%	2/3 66.7%
					Total *Microtus* spp. **n=15**	**4/15** **26.7%**	**7/15** **46.7%**
					Total rodents n=49	**8/49** **16.3%**	**22/49** **44.9%**

Table 4 (continued)

Area	Region	Site	Habitat	Month and year	Host species and numbers	*Babesia microti* I/N % infected	*Bartonella* spp. I/N % infected
Non-endemic for *D. reticulatus*	Mazovia, Central Poland	Niewiadów	Fallow lands	June and August 2017	*Myodes glareolus* n = 1	0/1 0%	1/1 100%
					***Apodemus agrarius* n = 11**	**5/11 45.5%**	**3/11 27.3%**
					Apodemus flavicollis n = 3	0/3 0%	3/3 100%
					Microtus oeconomus n = 19	15/19 78.9%	5/19 26.3%
					Total rodents n = 34	**20/34 58.8%**	**12/34 35.3%**
				Overall total	Rodents n = 83	28/83 33.7%	34/83 40.9%

In bold the host species/genera used as separate groups in statistical analyses

I—number of positive samples; N—number of examined samples

collected from *Microtus* spp. and the highest in ticks collected from *Apodemus* spp. (*Rickettsia* PRESENCE/ABSENCE × HOST SPECIES: $\chi^2_3 = 33.9$, P < 0.001). As only two ticks were positive for *B. canis*, no further analysis of this species was possible.

Host sex had significant effects on the frequency of detection of *B. microti* and *Rickettsia* in *D. reticulatus* ticks (*B. microti* PRESENCE/ABSENCE × HOST SEX: $\chi^2_1 = 4.76$, P = 0.029; *Rickettsia* PRESENCE/ABSENCE × HOST SEX: $\chi^2_1 = 8.09$, P = 0.004) and a similar trend was observed for *Bartonella* although it was not significant (Fig. 2b, NS). The frequency of PCR-positive samples was higher in both cases for ticks collected from male in comparison to female rodents.

Host age also had a significant effect on the frequency of detection of *B. microti* in *D. reticulatus* and similar trends in the same direction were noted for the detection of *Bartonella* and *Rickettsia* spp. (Fig. 2c; *B. microti* PRESENCE/ABSENCE × HOST AGE: $\chi^2_1 = 3.89$, P = 0.048). The frequency of PCR-positive samples was higher for ticks collected from adult rodents in comparison to juvenile ones.

Frequency of pathogen detection in ticks from infected and non-infected rodents

The influence of host infection status on detection of pathogen DNA in feeding ticks was analyzed for 49 rodents from Białobrzegi, for which both *D. reticulatus* ticks (Table 3) and the corresponding rodent host blood samples (Table 4) were available for testing for the presence of *Babesia* and *Bartonella* DNA. Comparison of the frequency of pathogen detection in ticks from infected and non-infected hosts is presented in Fig. 3a, b. Host infection had significant effect on detection of *B. microti* in ticks depending on tick stadium (*B. microti* TICK INFECTION × TICK STADIUM × rodent HOST INFECTION: $\chi^2_1 = 14.9$, P < 0.001). The DNA of *B. microti* was detected in 68.8% of nymphs collected from *B. microti*-positive rodents in comparison to 1.6% of nymphs collected from *B. microti*-negative rodents. However, the four *B. microti*-positive larvae were obtained only from uninfected hosts (Fig. 3a).

Bartonella spp. DNA was detected in 42% of nymphs and 57% of larval pools collected from *Bartonella*-positive rodents in comparison to 28% of nymphs and 11% of larvae collected from *Bartonella*-negative rodents (*Bartonella* TICK INFECTION × rodent HOST INFECTION: $\chi^2_1 = 9.09$, P = 0.003) (Fig. 3b).

The DNA of *B. canis* was detected in *D. reticulatus* nymphs collected from *M. glareolus* of unknown *Babesia* infection status and in a nymph from an uninfected *M. oeconomus*.

Discussion

The main finding of our study is the detection of *B. canis* in juvenile *D. reticulatus*, collected from voles that do not carry *B. canis*, hence supporting the occurrence of vertical transmission of the piroplasm under natural conditions. Additionally, through comparison of the frequency of successful pathogen detection in ticks and rodents (tick hosts) we have demonstrated clearly that the application of molecular techniques in this study has enabled detection of 'meal contamination' in ticks and that such contamination must be taken into account when evaluating the role of ticks as vectors of parasitic organisms.

To the best of our knowledge, this is the first field study in which *B. canis* has been detected in naturally infected juvenile *D. reticulatus* ticks. Recently, we have demonstrated transovarial transmission of *B. canis* from infected females to eggs and larvae under
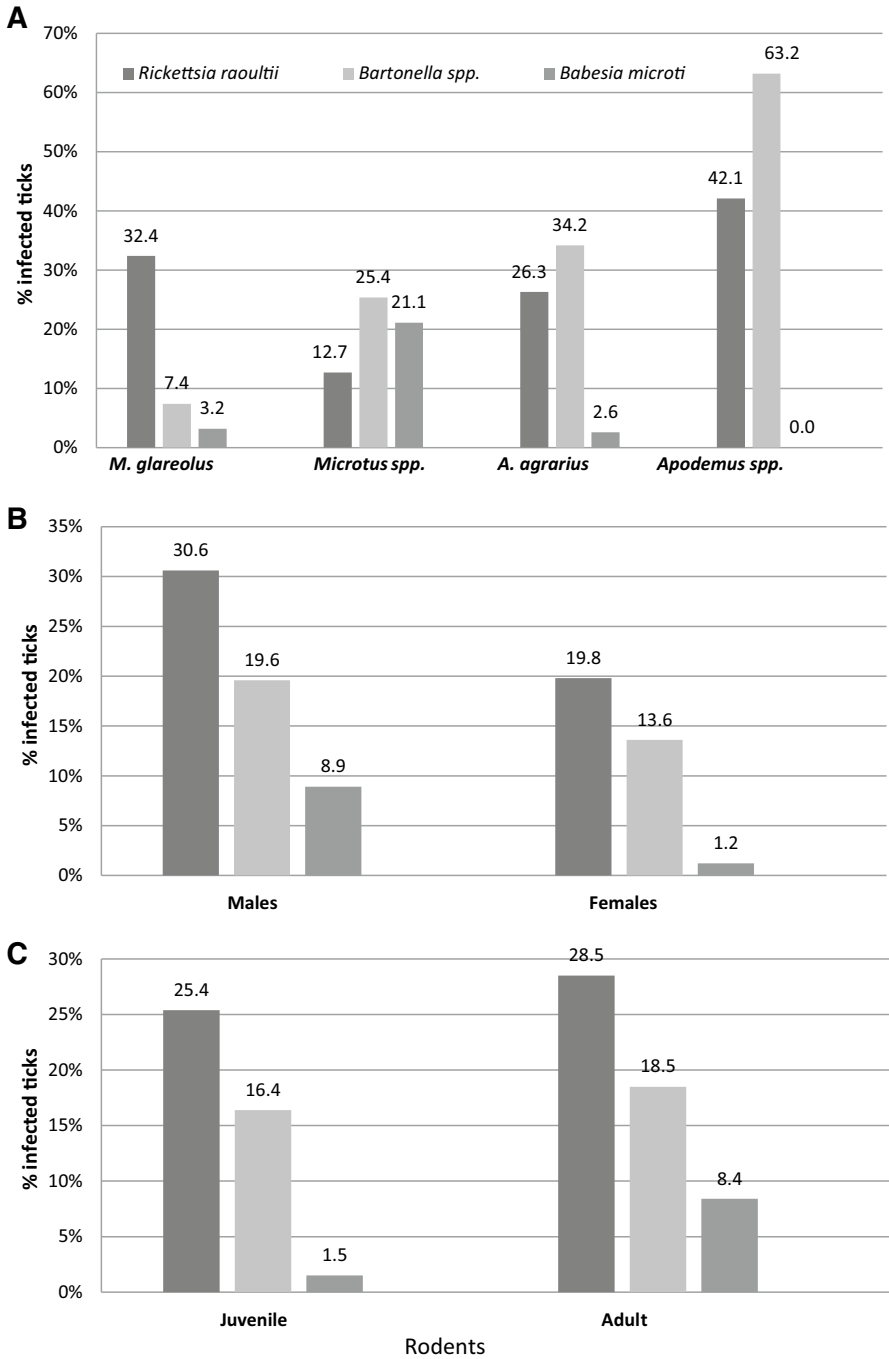
Fig. 2 Influence of host factors (**a** host species/genus; **b** host sex; **c** host age) on the frequency of pathogen detection in feeding ticks

laboratory conditions (Mierzejewska et al. 2018). However, these studies on nidicolous juvenile *D. reticulatus* ticks were not carried out under natural conditions. In our previous study on *D. reticulatus* collected from rodents we found only *B. microti* DNA (Welc-Faleciak et al. 2008b). In agreement with this earlier finding, here also the majority of *Babesia*-positive samples were shown to be *B. microti*. Interestingly, we have demonstrated also that rodent host factors (host species, age and infection status) have a significant effect on the frequency of detection of *B. microti* in ticks. Moreover, in agreement with previous findings (Pawelczyk et al. 2004; Welc-Faleciak et al. 2008b; Tołkacz et al. 2017) we have provided evidence that *Microtus* spp. constitute the main reservoir hosts of *B. microti* and are the major source of tick contamination with *B. microti* DNA.

If *D. reticulatus* constitutes a competent vector of *B. microti*, the frequency of DNA detection should be independent of rodent host factors, and should not correspond with the known effects of host species, age or sex on the prevalence of this pathogen in rodents (Pawelczyk et al. 2004; Tołkacz et al. 2017). Although it is widely accepted that *D. reticulatus* ticks are the main vectors of *B. canis* (notably by the link between the appearance of new canine babesiosis foci and the concurrent appearance of *D. reticulatus* in the same local environment; Matjila et al. 2005; Beelitz et al. 2012; Schaarschmidt et al. 2013; Medlock et al. 2017; de Marco et al. 2017), there are only a few papers reporting various rates of *B. microti*-positive questing adult *D. reticulatus* (Mierzejewska et al. 2015a; Wójcik-Fatla et al. 2015; Zając et al. 2017). Since we detected the DNA of *B. microti* in engorged nymphs, it seems reasonable to assume that a remnant of this *B. microti* DNA from this nymphal meal could be detectable later in questing adults. Blood residues are present in the gut of adult ticks after molting and are regularly used for the synthesis of adipose tissue (Sonenshine and Roe 2013). A role for *D. reticulatus* as an important vector for *B. microti* appears therefore to be questionable; especially as in this study we recorded the highest prevalence of *B. microti* in *M. oeconomus* from Niewiadów, where no feeding juvenile or questing *D. reticulatus* adult ticks have been found in recent years (Mierzejewska et al. 2016). In addition, in our recent study on *Microtus* spp. we found vertical transmission of *B. microti* in voles (from females to embryos and neonates or newborns), to be a common occurrence resulting in a high prevalence of infection in new generations without a contribution from arthropod vectors (Tołkacz et al. 2017). A similar recent study in *Peromyscus leucopus* (Tufts and Diuk-Wasser 2018) reached much the same conclusion.

Questing adult *D. reticulatus* ticks from the same geographical areas in Masuria and Mazovia as those sampled in the present study have shown a prevalence of *B. canis* DNA of 2.3% and 8.0% respectively, but *B. microti* DNA was found only in 1 adult tick from Masuria (0.2%; Mierzejewska et al. 2015a). Thus in conclusion, although we found a much higher rate of *B. microti*-positive than *B. canis*-positive juvenile *D. reticulatus*, we support a vector role of this tick species only for *B. canis*. *Babesia microti* detection was highly dependent on infections in rodents and hence more likely to be contamination from the infected rodents, rather than stages developing from earlier acquisition and involved in transmission.

The role of different tick species as vectors for *Bartonella* is still a hot topic in the scientific literature, with approximately equal numbers of scientists/papers supporting and rejecting a pivotal role for ticks as vectors (Angelakis et al. 2010; Telford and Wormser 2010; Billeter et al. 2008, 2012). There are numerous papers reporting various frequencies for the detection of *Bartonella* DNA in ticks (Angelakis et al. 2010; Bonnet et al. 2013; Maggi et al. 2018), including both *Bartonella* species typically encountered in rodents and *Bartonella* species of public health significance, such as *B. henselae* (Maggi et al. 2018; Földvári et al. 2016; Reye et al. 2013; Rar et al. 2005b). Despite these studies there are still
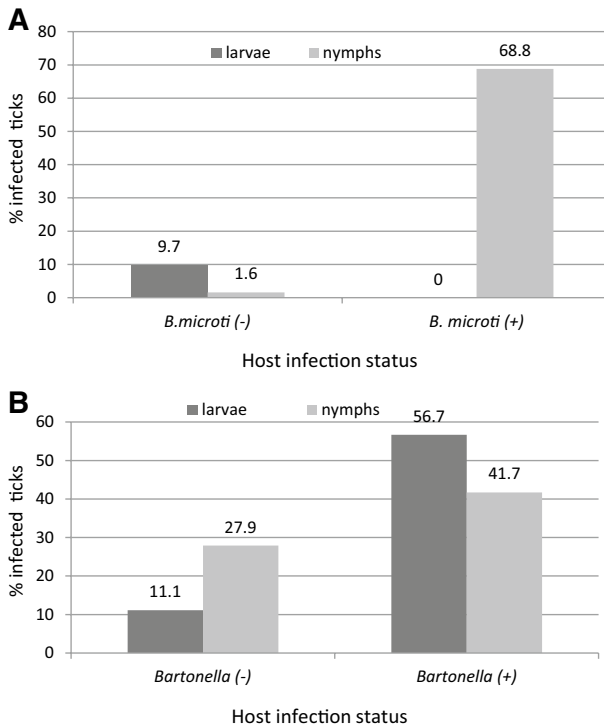
Fig. 3 Influence of host infection on the frequency of pathogen detection in feeding ticks. **a** For *Babesia microti*. **b** For *Bartonella* spp.

no unequivocally convincing experimental or epidemiological studies providing conclusive evidence that bartonellae are transmitted by ticks (Telford and Wormser 2010). Apparently, there is a weak association, or more often no association, between 'tick factors' (tick seasonality, tick foci, occurrence of borreliosis) and the occurrence of bartonellosis or seroconversion in humans (Telford and Wormser 2010; Zając et al. 2015; Muller et al. 2016). The majority of published findings on the detection of *Bartonella* in ticks is based on DNA detection (not on bacterial cultures) and these mostly report low prevalence (0.5–9%) in questing hard ticks, including *D. reticulatus* (Bonnet et al. 2013; Reye et al. 2013; Muller et al. 2016; Maggi et al. 2018). Such low prevalence of about 0.5–1% is not compatible with a key role for ticks of this species as vectors and should be interpreted more as accidental findings, without a crucial role in epidemiology. In our study on engorged *D. reticulatus* ticks, as for *B. microti*, the frequency of detection of *Bartonella* DNA was significant (18%) but also influenced by host factors, especially host species and infection status, and was the highest for ticks removed from the well-known *Bartonella* reservoir hosts-*Apodemus* spp. (Gutierrez et al. 2015; Paziewska et al. 2011), in which the prevalence of *Bartonella* infection was as high as 90%. Interestingly, in our previous study on *Bartonella* in *D. reticulatus* (feeding juvenile and questing adults) collected in Masuria, no positive ticks were recorded (Welc-Faleciak 2008), as also in a study in Hungary (Sréter-Lancz et al. 2006) and another in Serbia (Tomanovic et al. 2013). Again, *Bartonella* infections were prevalent in rodent hosts from Niewiadów, where no *D. reticulatus* were recorded.

Finally, all three species/genotypes of *Bartonella* identified in the present study have been previously found/described in rodents (Welc-Faleciak et al. 2008a; Paziewska et al. 2011; Buffet et al. 2013; Gutierrez et al. 2015; Tołkacz et al. 2018). Thus we conclude that the *Bartonella* DNA detected in feeding juvenile *D. reticulatus* in this study represents a case of typical 'meal contamination' and does not support a vector role of this tick species for these bacteria.

Rickettsia was the most common pathogen detected in juvenile *D. reticulatus* in this study and this high prevalence corresponds well with the high prevalence noted in questing adult *D. reticulatus* in Poland in our previous study (Mierzejewska et al. 2015a). All sequenced PCR products contained *R. raoultii*, which is also the most common bacterium (endosymbiont; Alberdi et al. 2012) found in *D. reticulatus* (Stańczak 2006; Silaghi et al. 2011; Tijsse-Klasen et al. 2013; Zając et al. 2017) and is transmitted vertically by the tick (Samoylenko et al. 2009). Interestingly, we found a significant influence of host species on the prevalence of *Rickettsia* in ticks, but in this case it might have been dependent more on the content of tick samples, especially on the number of larvae in pools rather than on host factors, as no rodent species are recognized as *R. raoultii* reservoirs. Another reason may be the natural dynamics of tick microbiota in relation to feeding status. A growing number of studies on the microbiome of ticks indicate high dynamic fluctuations/changes of microorganisms of different taxa in the course of the consecutive developmental and reproductive stages during the life-cycle of ticks (hatching, questing, feeding, molting, sperm or egg development) (Menchaca et al. 2013). It has been shown that some microorganisms are undetectable at times, even by molecular tools, depending on the tick's developmental stadium and that a blood meal can promote or inhibit the growth of some taxa, thus influencing their detection (Heise et al. 2010). Therefore, a recent uptake of blood by *D. reticulatus* larvae and nymphs may enhance the detectability of *R. raoulti* in ticks collected from rodents.

Conclusions

Vertical transmission of *B. canis* in *D. reticulatus* ticks was confirmed in the field. Additionally, we demonstrated that 'meal contamination' generates a confounding signal in molecular detection of pathogen DNA extracted from ticks collected from infected hosts and must be taken into account in evaluating the suitability of tick species as vectors of the respective pathogens.

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