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ORIGINAL ARTICLE

Prevalence of infections and co-infections with 6 pathogens in *Dermacentor reticulatus* ticks collected in eastern Poland

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Abstract

Occurrence of co-infections with various pathogens in ixodid ticks creates a risk of increased severity of tick-borne diseases in humans and animals exposed to bite of the ticks carrying multiple pathogens. Accordingly, co-infections in ticks were subject of numerous analyses, but almost exclusively with regard to *Ixodes ricinus* complex whereas potential tick vectors belonging to other genera were much less studied. Taking into consideration the role of *Dermacentor reticulatus* in the transmission of various pathogens, we carried out for the first time the comprehensive statistical analysis of co-infections occurring in this tick species. An attempt was made to determine the significance of the associations between 6 different pathogens occurring in *D. reticulatus* (Tick-borne encephalitis virus = TBEV, *Anaplasma phagocytophilum*, *Rickettsia raoultii*, *Borrelia burgdorferi* s. l., *Babesia* spp., *Toxoplasma gondii*), using 2 statistical methods: determination of Odds Ratios (ORs) and the Fisher's exact test. 634 questing *Dermacentor reticulatus* ticks (370 females and 264 males) were collected in 2011–2013 by flagging the lower vegetation in 3 localities in the area of Łęczyńsko-Włodawskie Lakeland, situated in the Lublin region of eastern Poland. The presence of individual pathogens was detected by PCR. Ticks were infected most often with *Rickettsia raoultii* (43.8%), less with TBEV (8.5%), and much less with *Babesia* spp., *Toxoplasma gondii*, *Borrelia burgdorferi* s.l., and *Anaplasma phagocytophilum* (2.5%, 2.1%, 1.6% and 1.1%, respectively). The locality-dependent variability proved to be significant for TBEV (χ^2=11.063; P=0.004) and *Toxoplasma gondii* (χ^2=11.298; P=0.0035), but not for other pathogens. Two hundred seventy (42.6%) of the examined ticks were infected only with a single pathogen, and 54 (8.5%) showed the presence of dual co-infections, each with 2 pathogens. The most common were dual infections with participation of *Rickettsia raoultii* (7.41%); next, those with participation of the TBEV (5.21%), *Toxoplasma gondii* (1.58%), *Borrelia burgdorferi* s.l. (1.26%), *Anaplasma phagocytophilum* (0.95%), and *Babesia* spp. (0.63%). On the total number of 15 possible associations, in 9 cases co-infections occurred whereas in 6 cases they were not detected. The most noteworthy were positive co-infections with the participation of TBEV, which proved to be weakly significant (0.05<P<0.1) in associations with *Toxoplasma gondii* and *Anaplasma phagocytophilum*, with Odds Ratios over 3.3 and 4.4, respectively. The values of Odds Ratios exceeded 3.0 also at the co-infections of *Rickettsia raoultii* with *B. burgdorferi* s.l., and *T. gondii* with *Babesia* spp., but these associations did not attain a significance level. The co-infections of *Rickettsia raoultii* with *Babesia* spp. appeared not to be significant (0.05<P<0.1) with OR below 0.3. In conclusion, co-infections with various pathogens in *D. reticulatus* ticks seem to be relatively rare and mostly not significant.

Key words

Dermacentor reticulatus, co-infections, TBE virus, *Anaplasma phagocytophilum, Rickettsia raoultii*, *Borrelia burgdorferi* sensu lato, *Babesia* spp., *Toxoplasma gondii*

INTRODUCTION

Hard ticks (Ixodidae) transmit a range of viruses, bacteria and protozoa pathogenic for humans and animals. The occurrence of co-infections (multiple infections with pathogens of different genera) or mixed infections (multiple infections with pathogens of the same genus), could be of medical significance because of increased severity of pathologic symptoms [1]. The prevalence of co-infections has been studied mostly within *Ixodes ricinus* complex [2, 3, 4, 5], whereas potential tick vectors belonging to the other genera were much less studied.

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Dermacentor reticulatus (Fabricius, 1794) is an Eurasian tick species, known as marsh tick or ornate dog tick. This species is regarded as the second most important vector of tick-borne pathogens in Europe, next to *I. ricinus* [6, 7, 8]. *D. reticulatus* has been classified by Estrada-Peña and Jongejan [9] as a pathogen-transmitting tick commonly reported on humans and ungulates.

The area of occurrence of *D. reticulatus* extends from Portugal in the West to Western Siberia in the East and comprises the Western-European and the Eastern tick populations. Both populations are separated by a gap not inhabited by *D. reticulatus*, which is shrinking because of rapid expansion of marsh ticks to new areas. The spatial expansion of *D. reticulatus* in Poland is associated with the increase of the number of ticks which, in some areas of central and eastern Poland, became dominant over *I. ricinus* [10,

11, 12, 13, 14, 15, 16, 17]. These findings indicate a growing significance of marsh ticks as potential vectors of tick-borne diseases [8, 16].

The role of *D. reticulatus* in the circulation and transmission of tick-borne pathogens remains poorly known. Nevertheless, *D. reticulatus* has been demonstrated to harbour various tick-borne pathogens of humans and/or animals, such as tick-borne encephalitis virus (TBEV), Omsk haemorrhagic fever virus, Kemerovo virus, *Anaplasma phagocytophilum*, *Anaplasma marginale*, Spotted Fever Group Rickettsiae (SFGR), such as *Rickettsia raoultii* and *Rickettsia slovaca*, *Borrelia burgdorferi* sensu lato (s.l.), *Francisella tularensis* and *Francisella*-like endosymbionts, *Coxiella burnetii*, *Bartonella* spp., *Babesia canis*, *Babesia caballi*, *Babesia microti*, *Babesia divergens*, *Babesia bigemina,* and *Theileria equi* [7, 8, 18–32].

The best documented is the important role of *D. reticulatus* as a vector of the babesiosis in dogs caused by *Babesia canis* [18, 33, 34]. It is also regarded as a vector of *Rickettsia raoultii* and *Rickettsia slovaca* causing tick-borne lymphadenopathy (TIBOLA) in humans. Földvári et al. [26] has demonstrated recently a direct relationship between the bite of a man by *D. reticulatus* and development of TIBOLA caused by *Rickettsia raoultii*. The role of this tick species in the transmission of some other pathogens in domestic animals and humans was also confirmed for *Babesia caballi, Theileria equi*, *Anaplasma marginale*, and Omsk haemorrhagic fever virus [18]. The role of *D. reticulatus* as a vector of other pathogens is less known, but seems probable with relevance to TBEV, and less probable with relevance to *Francisella tularensis*, *Coxiella burnetii* and *Babesia microti* [8, 18, 24, 25, 29–31, 35].

In contrast to the *Ixodes ricinus* complex, the prevalence and significance of co-infections occurring in *D. reticulatus* has not been extensively studied. Taking into consideration the possible role of this tick species in the transmission of tick-borne pathogens, we carried out for the first time a comprehensive analysis of co-infections occurring in *D. reticulatus*. We attempted to determine the character of the associations between 6 different pathogens occurring in this tick species, utilizing 2 statistical methods used by Nieto and Foley [4] and by our group [36] for assessment of the significance of co-infections occurring in *Ixodes* ticks: determination of Odds Ratios (ORs) and the Fisher's exact test.

The presented study includes into the analysis of co-infections with 5 pathogens described earlier as indigenous for *D. reticulatus* (TBEV, *Anaplasma phagocytophilum*, *Rickettsia* spp., *Borrelia burgdorferi* s.l., *Babesia* spp.), adding as a sixth pathogen, an apicomplexan parasite *Toxoplasma gondii* which, until recently, has not been regarded as a tick-borne pathogen. This protozoan occurs in wide range of vertebrate hosts, spreads by the oral route, and completes its life cycle in cat's intestine [37]. However, as *T. gondii* DNA has been detected already in *Dermacentor reticulatus* ticks from eastern Poland [38], from *Ixodes ricinus* ticks from various areas of Poland [39–41], and recently also from *Haemaphysalis longicornis* ticks in China [42], the tick-borne spread of this parasite cannot be excluded, and we have therefore included it into the current analysis as a novel element.

MATERIALS AND METHOD

Collection of ticks. A total of 634 adult questing *D. reticulatus* ticks (370 females and 264 males) were collected from spring to autumn in 2011–2013 in 3 localities situated in the Lublin province (eastern Poland): Ostrów Lubelski, Parczew and Włodawa. Ticks were collected by dragging a woolen flag over the lower vegetation and litter along the paths and edges of deciduous and mixed forests, and kept alive until further investigation.

DNA and RNA isolation. All ticks were examined individually and after collection each tick was cut in half. From one part of the tick, after homogenization, total DNA was isolated by boiling in 0.7 M ammonium hydroxide [43] and stored at -20 C for further analysis. The second half of tick was crushed in liquid nitrogen, homogenized with a syringe needle and suspended in buffer containing guanidine isothiocyanate (GITC), inhibiting RNase enzyme. Total RNA was extracted using RNeasy Mini Kit (Qiagen, USA) according to the producer's instruction. The concentration of DNA and RNA in the isolates was determined with the NanoDrop ND1000 Spectrophotometer (USA). DNA concentration ranged from 240–460 ng/µl and RNA concentration ranged from 15–60 ng/µl.

Detection of pathogens by PCR. Using polymerase chain reaction (PCR), DNA isolates were examined for the presence of *A. phagocytophilum*, *Rickettsia* spp., *B. burgdorferi* s.l., *Babesia* spp., and *T. gondii,* while RNA isolates were examined for the presence of TBEV. All amplifications were carried out in C1000 Thermal Cycler (BioRad, USA).

Detection of *A. phagocytophilu*m was performed according to Massung et al. [44] using amplification by PCR and confirmatory re-amplification by nested-PCR. Each PCR reaction was carried out in a 25 µl reaction volume which contained the following mix of reagents: 1.25 U *Taq* DNA polymerase (Qiagen, USA), 1×PCR buffer containing 15 mM $MgCl_2$, 2.5 µl 2 mM dNTP (final concentration 0.2 mM) (Thermo Scientific), 1.25 µl 10 µM each of primer for the first amplification (ge3a/ge10) or 0.5 µl 10 µM each of primer for nested-PCR (ge9/ge2), 2.5 µl of isolated DNA and nuclease-free water (Applied Biosystems, USA). For nested-PCR reaction 1 µl from the primary PCR was added. The size of the amplified DNA fragment was 546 base pairs (bp). Products of amplification were identified in 2% agarose gel, after electrophoresis in standard conditions and staining with ethidium bromide solution (2 µg/ml). DNA isolated from antigen *A. phagocytophilum* on the substrate slide (Focus Diagnostic, CA, USA) was used as a positive control and nuclease-free water as negative control.

Rickettsia spp. detection was performed as described previously by Wójcik-Fatla et al. [29] using primers RpCS.887p and RpCS.1258n specific for a gene *gltA* encoding the citrate synthase [45]. DNA isolated from antigen *Rickettsia* spp. on the substrate slide (Fuller Laboratories, CA, USA) was used as a positive control.

The presence of *B. burgdorferi* sensu lato (s. l.) in ticks was evaluated with use of primers FLA1 and FLA2 [46, 47] specific for *fla* gene. Genus of *B. burgdorferi* s.l. from positive samples was confirmed by nested PCR, according to method described by Wójcik-Fatla et al. [36].

Detection of *Babesia* spp. has been performed according to methods described previously [31], using the primers described by Persing et al. [48] and by Hilpertshauser et al. [49].

Detection of *T. gondii* DNA was based on amplification of the B1 fragment gene according to Grigg and Boothroyd [50], with modification described previously by Wójcik-Fatla et al. [38].

Detection of TBEV was carried out using nested RT-PCR (reverse transcription-polymerase chain reaction) with primers specific for the 5'-terminal non-coding region, as described by Schrader and Süss [51] with some modifications [24].

DNA sequencing. Sequencing of positive samples was performed with ABI PRISM 310 Genetic Analyzer using Abi Prism Big Dye Terminator v. 3.1. Cycle Sequencing Kits and Big Dye XTerminator Purification Kit (Applied Biosystems). The results were compared with sequences in the GenBank database using the BLAST server.

Statistical analysis. The results concerning prevalence of individual pathogens in examined ticks were analyzed by χ^2 test and Student's t-test, using the STATISTICA v. 6.0 package (Statsoft, Tulsa, OK, USA). The significance of co-infections was assessed with 2 methods: by the Fisher's exact test using GraphPad software [52] and by Odds Ratio calculation using MedCalc® software [53]. The value of $P<0.05$ was considered as significant and the value of $0.05<P<0.1$ as weakly significant. The test provided an answer to the questions whether the observed prevalence of co-infections was greater or smaller than expected by chance, and whether this relationship was significant ($P<0.05$), weakly significant ($0.05<P<0.1$) or not significant ($P>0.05$).

RESULTS

Prevalence of infections with 6 pathogens among *Dermacentor reticulatus* ticks. The proportion of ticks infected with *Rickettsia* spp. was large (43.8%, on the average), much greater compared to other pathogens. As much as 8.5% of examined ticks were infected with the TBE virus. The infection rates with *Babesia* spp., *Toxoplasma gondii*, *Borrelia burgdorferi* s.l., and *Anaplasma phagocytophilum* were smaller, amounting to 2.5%, 2.1%, 1.6% and 1.1%, respectively (Tab. 1).

Sequencing analysis of PCR positive samples confirmed the 100% homology with sequences deposited in GenBank – for *Anaplasma phagocytophilum* [Accession No. CP015376.1], Tick-borne encephalitis virus [FJ572210.1], *Toxoplasma gondii* [LN714499.1], for *Rickettsia* spp. the comparison evidenced *R. raoultii* [KU310590.1], and for *B. burgdorferi* s.l positive samples – analysis confirmed *B. afzelii* [KR782215.1]. Among *Babesia* spp. positive samples, 12 were confirmed as *B. microti* [AB085191.1] and 4 as *B. canis* [KM111283.1].

The infection rate with *T. gondii* was significantly greater in males for total examined tick population ($P=0.0448$) and for the Włodawa locality ($P=0.0159$). Similarly, males were infected significantly more often with *Babesia* spp. in Parczew ($P=0.0242$) and in total tick population, where the difference was only weakly significant ($P=0.0817$). By contrast, female ticks in Parczew were infected significantly more often with *B. burgdorferi* s.l. ($P=0.0315$). For TBEV, *Anaplasma phagocytophilum*, and *Rickettsia raoultii*, no significant differences were found between infection rates in males and females.

The greatest locality-dependent variability was found for TBE virus, having been significant for females ($\chi^2=8.609$; $P=0.0135$), males ($\chi^2=8.973$; $P=0.0113$), and total tick population ($\chi^2=11.063$; $P=0.004$). For *Toxoplasma gondii*, this variability was significant for males ($\chi^2=12.056$; $P=0.0024$) and total tick population ($\chi^2=11.298$; $P=0.0035$), while for *Borrelia burgdorferi* s.l. it was significant only for males ($\chi^2=6.100$; $P=0.0474$). No significant locality-dependent variability was found for *A. phagocytophilum*, *Rickettsia raoultii*, and *Babesia* spp.

Co-infections with multiple pathogens and their significance. Of the total 634 *D. reticulatus* specimens examined, 310 (48.9%) were not infected with any of the 6 pathogens tested, 270 (42.6%) were infected with a single

Table 1. Prevalence of 6 pathogens in the population of *Dermacentor reticulatus* ticks collected in eastern Poland

Locality	Stage, No. (N)		TBE virus	*Anaplasma phagocytophilum*	*Rickettsia raoultii*	*Borrelia burgdorferi* s.l.	*Babesia* spp.	*Toxoplasma gondii*
Ostrów Lubelski	Females	81	10 (12.3%)	0	31 (38.3%)	0	2 (2.5%)*	1 (1.2%)**
	Males	67	11 (16.4%)	0	27 (40.3%)	0	1 (1.5%)*	3 (4.5%)**
	Total	148	21 (14.2%)	0	58 (39.2%)	0	3 (2.0%)*	4 (2.7%)**
Parczew	Females	147	8 (5.4%)	3 (2.0%)	64 (43.5%)	5 (3.4%)	1 (0.7%)*	0
	Males	135	5 (3.7%)	2 (1.5%)	62 (45.9%)	0	7 (5.2%)*	0
	Total	282	13 (4.6%)	5 (1.8%)	126 (44.7%)	5 (1.8%)	8 (2.8%)*	0
Włodawa	Females	142	13 (9.2%)	1 (0.7%)	61 (43.0%)	3 (2.1%)	3 (2.1%)	3 (2.1%)
	Males	62	7 (11.3%)	1 (1.6%)	33 (53.2%)	2 (3.2%)	2 (3.2%)	6 (9.7%)
	Total	204	20 (9.8%)	2 (1.0%)	94 (46.1%)	5 (2.5%)	5 (2.5%)	9 (4.4%)
All localities	Females	370	31 (8.4%)	4 (1.1%)	156 (42.2%)	8 (2.2%)	6 (1.6%)	4 (1.1%)
	Males	264	23 (8.7%)	3 (1.1%)	122 (46.2%)	2 (0.8%)	10 (3.8%)	9 (3.4%)
	Total	634	54 (8.5%)	7 (1.1%)	278 (43.8%)	10 (1.6%)	16 (2.5%)	13 (2.1%)

In each space there is shown the number of infected ticks and percent of the total in parentheses.
* results previously published in: Wójcik-Fatla A, Zając V, Sawczyn A, Cisak E, Dutkiewicz J.: *Babesia* spp. in questing ticks from eastern Poland: prevalence and species diversity. Parasitol Res. 2015 Aug; 114(8):3111–6. doi: 10.1007/s00436–015–4529–5.
** results previously published in: Wójcik-Fatla A, Sroka J, Zając V, Sawczyn A, Cisak E, Dutkiewicz J.: *Toxoplasma gondii* (Nicolle et Manceaux, 1908) detected in *Dermacentor reticulatus* (Fabricius) (Ixodidae). Folia Parasitol (Praha). 2015 Sep 18;62. pii: 2015.055. doi: 10.14411/fp.2015.055.

Table 2. Co-infections in *Dermacentor reticulatus*: prevalence and significance

TBE virus (TBEV)	**Versus *A. phagocytophilum*** C: 2 (0.32%) OR: 4.4231 95% CI: 0.84–23.36 P_{OR}: 0.0799* P_{Fisher}: 0.1134	**Versus *Rickettsia raoultii*** C: 27 (4.26%) OR: 1.3108 95% CI: 0.75–2.29 P_{OR}: 0.3420 P_{Fisher}: 0.3902	**Versus *B. burgdorferi* s. l.** C: 1 (0.16%) OR: 1.1971 95% CI: 0.15–9.63 P_{OR}: 0.8657 P_{Fisher}: 0.5922	**Versus *Babesia* spp.** NC
Anaplasma phagocytophilum	**Versus *Rickettsia raoultii*** C: 4 (0.63%) OR: 1.7178 95% CI: 0.38–7.74 P_{OR}: 0.4811 P_{Fisher}: 0.7050	**Versus *B. burgdorferi* s. l.** NC	**Versus *Babesia* spp.** NC	**Versus *Toxoplasma gondii*** NC
Rickettsia raoultii	**Versus *B. burgdorferi* s. l.** C: 7 (1.10%) OR: 3.0394 95% CI: 0.78–11.86 P_{OR}: 0.1096 P_{Fisher}: 0.1141	**Versus *Babesia* spp.** C: 3 (0.47%) OR: 0.2878 95% CI: 0.08–1.02 P_{OR}: 0.0537# P_{Fisher}: 0.0437##	**Versus *Toxoplasma gondii*** C: 6 (0.95%) OR: 1.098 95% CI: 0.37–3.31 P_{OR}: 0.8656 P_{Fisher}: 1.000	
***Borrelia burgdorferi* s. l.**	**Versus *Babesia* spp.** NC	**Versus *Toxoplasma gondii*** NC		
***Babesia* spp.**	**Versus *Toxoplasma gondii*** C: 1 (0.16%) OR: 3.3667 95% CI: 0.41–27.59 P_{OR}: 0.2580 P_{Fisher}: 0.2850			
Toxoplasma gondii	**Versus TBE virus** C: 3 (0.47%) OR: 3.3529 95% CI: 0.89–12.57 P_{OR}: 0.0728* P_{Fisher}: 0.0907*			

C=Number of co-infections and percent related to the total tick population examined (634 specimens);
OR=Odds Ratio, indicating the ratio of infections with pathogen "A" in ticks infected also with pathogen "B" to the infections with pathogen "A" in ticks not harbouring pathogen "B";
95% CI=95% confidence intervals for OR;
P_{OR}=Probability of co-infection assessed by Odds Ratio;
P_{Fisher}=Probability of co-infection assessed by the Fisher's exact test.
*relationship weakly significant (0.05<P<0.1);
#-##=relationship inversely significant (more infections with pathogen "A" in ticks not harboring pathogen "B"): #0.05<P<0.1, ##P<0.05.
NC=No co-infections found.

pathogen, and 54 (8.5%) showed the presence of dual co-infections, each with 2 pathogens. No simultaneous infections with 3 or more pathogens were found. The occurrence of dual co-infections and the assessment of their significance performed with 2 methods are presented in Table 2.

As seen in Table 2, the most common were dual infections with participation of *Rickettsia raoultii* (7.41%), followed by those with participation of the TBE virus (5.21%). Less common were dual infections with participation of *T. gondii*, *B. burgdorferi* s.l., *A. phagocytophilum*, and *Babesia* spp. (1.58%, 1.26%, 0.95%, and 0.63%, respectively). Of the total number of 15 possible associations, in 9 cases co-infections occurred, whereas in 6 cases they were not detected (Tab. 2). The most noteworthy were positive co-infections with participation of TBE virus which proved to be weakly significant (0.05<P<0.1) at associations with *T. gondii* and *A. phagocytophilum* (Tab. 2). The Odds Ratios were respectively over 3.3 and 4.4, respectively, which means that the probability of infection with TBE virus (TBEV) was 3–4 times greater at the presence of the above-mentioned pathogens. The values of Odds Ratios exceeded 3.0 also at the co-infections of *Rickettsia raoultii* with *B. burgdorferi* s.l., and *T. gondii* with *Babesia* spp., but these associations did not attain a significance level. It is interesting that the co-infections of *Rickettsia raoultii* with *Babesia* spp. appeared to be negatively correlated with OR below 0.3, which means that the infections with bacteria of the species *R. raoultii* are less common at the presence of *Babesia* protozoans. In conclusion, co-infections with various pathogens in *D. reticulatus* ticks seem to be relatively rare and mostly not significant.

DISCUSSION

So far, there is only scarce information on co-infections with various pathogens in *Dermacentor reticulatus,* and the presented study, revealing 8.5% of dual infections in this tick species, is the first attempt at statistical assessment of this problem, based on a greater number of specimens examined. Until recently, the only statistical evaluation of these co-infections had been performed by Tomanović et al. [28] in Serbia, who studied the prevalence of 9 microorganisms in 53 *D. reticulatus* specimens, and detected only 2 types of dual infections: between *Coxiella burnetii* and *Francisella*-like endosymbiont (FLE), and between *Babesia canis* and FLE. The latter association showed a significantly higher prevalence rate than expected. Reye et al. [27] found co-infections in 3 out 226 *D. reticulatus* ticks (1.3%) which were collected in Belarus and tested for the presence of 7 pathogens. This value, which is 6.5 times lower compared to the current study, concerned in all cases co-infections between *B. burgdorferi* s.l. and *Rickettsia* spp. Similarly, a co-infection value 2.8 times lower compared to the current study was reported Mierzejewska et al. [30] who examined the Eastern and Western populations of the adult *D. reticulatus* ticks collected in Poland for the presence of 4 pathogens (*Rickettsia* spp., *Babesia* spp., *B. burgdorferi* s. l., TBEV), and found 3.0% of dual infections in the Eastern population (2.7% of *R. raoultii* with *B. canis*, and 0.3% of *R. raoultii* with TBEV), while in the Western population no such infections were found. The smaller value of co-infections reported also Ionita et al. [54], who found that 5.3% of *D. reticulatus* ticks collected from dogs in Romania, were simultaneously infected with *Rickettsia raoultii* and *Babesia canis*.

Among the pathogens determined in the course of the present work in *D. reticulatus*, the greatest prevalence showed *Rickettsia raoultii* causing spotted fever and tick-borne

encephalitis virus (TBEV). Taking into consideration the detected co-infections, the greatest risk is posed by TBEV, the only pathogen that revealed a weakly significant (0.05<P<0.1) positive association with *Anaplasma phagocytophilum* and *Toxoplasma gondii*. In particular, the co-infection of TBEV and *T. gondii* could be of clinical importance because both pathogens are neurotropic. Considering the significant locality-dependent variability of TBEV, its distribution in eastern Poland most probably has a focal character. *Rickettsia raoultii* showed only a negative association with *Babesia* spp., which means that the probability of the tick infection with *Babesia* spp. is more than 3 times smaller in the presence of *R. raoultii*.

The frequency of co-infections found in the presented study in *D. reticulatus* (8.5%) is essentially lower compared to analogous co-infections reported for *Ixodes ricinus* complex, although usually in the latter case co-infections involving less than 6 pathogens were analyzed. Thus, Steiner et al. [55] found 45% dual and triple infections in *I. scapularis* ticks which were tested in the USA for the presence of *B. burgdorferi* s.l., *A. phagocytophilum*, *Babesia* spp., and the rickettsial endosymbiont. Similarly, Tokarz et al. [56] found 38.1% dual and triple infections in *I. scapularis* ticks which were examined in New York State, USA, for the presence of *B. burgdorferi* s.l., *Borrelia miyamotoi*, *A. phagocytophilum*, *Babesia microti*, and Powassan virus. In three European studies performed in France [57], Luxembourg [58] and Belarus [27], where *I. ricinus* ticks were tested with a greater number of pathogens (6, 7 and 7, respectively), co-infections frequencies 16.3% (twice as many as in present study), 3.2% (2.7 times less than in the present study) and 3.4% (2.5 times less than in the present study) were respectively found.

We did not observe in *D. reticulatus* any dual infections with *B. burgdorferi* s.l. *and A. phagocytophilum*, with *B. burgdorferi* s.l. and *Babesia* spp., and with *A. phagocytophilum* and *Babesia* spp. Such co-infections were also not detected by other authors who examined *D. reticulatus* for the presence of a greater number of pathogens in Belarus [27], Serbia [28], Russia [20], and Poland [59], while in most studies on *I. ricinus* complex, these co-infections are usually reported and analyzed. Thus, Belongia [2] and Swanson et al. [1] report in their review articles the prevalence of dual co-infections between *B. burgdorferi* s. l. and *A. phagocytophilum* or *Babesia microti*/*B. divergens,* ranging between 1.0–1.3% for *I. pacificus*, between 1.0–28.2% for *I. scapularis*, between 0–13.4% for *I. ricinus*, and between 0.5–1.2% for *I. persulcatus*. Alekseev et al. [60] found that in *I. persulcatus* ticks collected in Russia, the infection with *B. microti* occurred only in a significant association with *B. burgdorferi* s.l., which resulted in a more severe and longer-lasting disease in humans attacked by the ticks. In *Ixodes ricinus* ticks collected in various regions of Poland, the prevalence of co-infections between *B. burgdorferi* s.l. and *A. phagocytophilum* ranged from 0.9–8.3%, between *B. burgdorferi* s.l. and *B. microti* from 0.1–0.6%, and between *A. phagocytophilum* and *B. microti* from 1.05–2.0%, while the frequency of triple infections between these 3 pathogens ranged from 0–0.06% [61]. In the Lublin Region, co-infections between *B. burgdorferi* s.l. and *A. phagocytophilum*, and between *A. phagocytophilum* and *B. microti* occurred in *I. ricinus* significantly more often than expected (P<0.001) [62]. Recently, Prusinski et al. [63] reported the average rates of co-infections in adult *I. scapularis* ticks collected in the USA as 6.3% for *B. burgdorferi* and *A. phagocytophilum*, 1.5% for *B. burgdorferi* and *B. microti*, and 0.6% for *A. phagocytophilum* and *B. microti*. The co-infections occurred more frequently than expected by chance (P<0.0001).

In the presented study, when co-infections with the participation of *Rickettsia raoultii* were excluded, the prevalence of remaining co-infections was only 1.1%, and single infections ranged from 1.1% – 8.4%. These relatively low results compared with infection and co-infection rates within *I. ricinus* could be connected with the shortened *D. reticulatus* life-cycle, where nymphs are active only for one month and there is no opportunity for co-feeding larvae and nymphs [26]. To the best our knowledge, the transovarial and transstadial transmission of the investigated pathogens has not been confirmed within the *D. reticulatus* population, with the exception of transovarial transmission of the TBEV via eggs from an infected adult females to offsprings [64]. Pathogens circulation is possible by transfer between hosts and vectors, but regarding the ability of adult *D. reticulatus* to survive in nature without hosts for even 3–4 years [26], this tick species seems to be a less competent reservoir and vector than *I. ricinus*.

Little is known about pathogen-pathogen and pathogen-tick interactions. Concerning the transmission of *B. burgdorferi,* the OspA protein seems to be the key agent responsible for the growth of spirochetes in ticks. The gene *TROSPA* coding tick receptor for OspA is strongly expressed in larvae and nymphs, compared to adult ticks [65]. Antunes et al. [66] confirmed that receptor TROSPA was over-expressed in *Rhipicephalus* tick after *Babesia bigemina* infection, and may be involved in *Babesia*–tick interactions. To the best of our knowledge, there is no study confirming the role of TROSPA receptor in *D. reticulatus* ticks, which may explain the lower prevalence of *B. burgdorferi* in *D. reticulates,* compared to *I. ricinus*. Studies by Moniuszko et al. [67] concerning interactions between pathogens in ticks confirmed that *B. burgdorferi* s.s. increased short-term replication of *Ehrlichia ruminantium* and Semliki Forest virus, while these pathogens did not affect replication of *Borrelia*. On the other hand, a study by Levin and Fish [68] on mice and *I. scapularis* showed no evidence of interaction between *B. burgdorferi* and *A. phagocytophilum,* and indicated that transmission of both pathogens was independent, the same as in case of a single bacterium.

According to some authors, there are a large number of factors contributing to the geographical spread of *D. reticulatus*, such as climate change, increasing wildlife population and more fallow lands [26]. The role of *D. reticulatus* ticks in pathogen circulation in the natural environment may increase within the next few years, when some co-infections occurring in *D. reticulatus* could become important, both from the medical and veterinary viewpoint.

CONCLUSION

In conclusion, the co-infections with various pathogens occurred in *D. reticulatus* less often than in the ticks of the *Ixodes ricinus* complex, although some of them could be important from the medical and veterinary viewpoint. Among the co-infections present in *D. reticulatus*, the most significant seem to be those with the participation of TBEV, while of no importance are those characteristic for

the *Ixodes ricinus* complex: between *B. burgdorferi* s.l. and *A. phagocytophilum*, between *B. burgdorferi* s.l. and *Babesia* spp., and between *A. phagocytophilum* and *Babesia* spp.

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