Molecular detection of tick-borne pathogens in ticks collected from pets in selected mountainous areas of Tatra County (Tatra Mountains, Poland)

Anna Kocoń, Marek Asman, Magdalena Nowak-Chmura, Joanna Witecka, Małgorzata Kłyś & Krzysztof Solarz

The mountainous and foothill areas, in which the city of Zakopane, the capital of Tatra County, is located are characterized by continuous weather changes, lower air temperature, persistent snow cover, and poorer vegetation than in the lowlands. *Ixodes ricinus* and *Ixodes hexagonus* are vectors of tick-borne diseases and play an important role in the persistence of tick-borne diseases. The aim of the study was to determine the risk of exposure of domestic cats and dogs to the attacks of ixodid ticks, to tick-borne infections with *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Babesia microti* and *Toxoplasma gondii* in the city of Zakopane and the surrounding area. In 2017–2018 ticks were collected from a total of 10 domestic cats and 88 domestic dogs. Selected pathogens of tick-borne diseases were detected by PCR and nested PCR. The study material contained 119 *I. ricinus* and 36 *I. hexagonus*. The molecular examinations showed the presence of *A. phagocytophilum* in 3.8%, *B. microti* in 24.5% and *T. gondii* in 4.5% of the all ticks. In addition, in the study area, there is a high potential risk of tick-borne infection by *B. microti*, and a low potential risk of exposure to *A. phagocytophilum* and *T. gondii* infection.
Due to the great medical and veterinary importance of ticks and close contact between human and domestic animals, an attempt has been made to determine the exposure of cats and dogs to tick infection, as well as to a potential tick-borne infection with B. burgdorferi s. l. and A. phagocytophilum, as well as to the infection of Babesia microti and Toxoplasma gondii in the selected areas of Tatra County. The approach of gathering data on the distribution of (zoonotic) vector-borne diseases through a veterinary survey is consistent with the ‘One Health’ concept.

Materials and methods
Zakopane, is the highest located city in Poland (49° 18’ N; 19° 57’ E), situated in the Podtatranski Trench, between the Tatra Mountains and the Beskids, in one of the largest mountain ranges in Europe—the Carpathian Mountains. The urban part stretches from 750 to 1000 m a.s.l., the administrative boundaries of the city contain a part of the Tatra Mountains with the highest point—the peak of Swinica (2301 m a.s.l.), as well as Gubalowka (1120 m a.s.l.), and the central point of Zakopane is located at an altitude of 838 m a.s.l.

The research material was obtained from domestic cats and dogs in 2017–2018 from March to September in cooperation with the veterinary clinic in Zakopane. The ticks were collected from animals using tweezers and placed in tubes with 70% ethyl alcohol. In addition, after the collection, an original form was completed with the following information: date of collection, animal breed, sex, age and the city of collection. Then, individual ticks were determined for genus, species and developmental stage. The keys by Siuda’ and Nowak-Chmura’ were used to identify the ticks. Next, molecular testing for the presence of selected pathogens was performed. DNA was isolated from 155 ticks using the ammonium hydroxide method. Then, the concentration was measured spectrophotometrically at the wave length of 260/280 nm.

Results

Statistical analysis was performed using CSS-Statistica for Windows 10. Statistical significance was accepted at a p value of less than 0.05. The results were analysed using an $\chi^2$ test.

Ethical approval. We declare that all testing methods have been carried out in accordance with the relevant guidelines and regulations. We declare that all experimental protocols have been approved by the Medical University of Silesia in Katowice and Pedagogical University in Cracow.

Informed consent. Each dog and cat owner has agreed to collect material (ticks) from their animals and give informed consent to publish the results of the collected material.

Each tick was collected from an individual animal using tweezers and placed in a tube with 70% ethyl alcohol. Then, an original form was completed with the following information: date of collection, animal breed, sex, age and the city of collection. Each cat and dog owner has agreed to collect material (ticks) from their animals and give informed consent to publish the results of the collected material.

Results

A total of 155 ticks were collected from 10 domestic cats and 88 domestic dogs. I. ricinus was the dominant tick species, with 119 females collected from 90 animals, including 8 cats and 82 dogs. 36 I. hexagonus ticks were also collected, including 15 larvae, 13 nymphs and 8 females. The presence of this tick species has been demonstrated in 8 animals, including 2 cats and 6 dogs. The infestation by I. ricinus was the dominant tick species, with 119 females collected from 90 animals, including 8 cats and 82 dogs. 36 I. hexagonus ticks were also collected, including 15 larvae, 13 nymphs and 8 females. The presence of this tick species has been demonstrated in 8 animals, including 2 cats and 6 dogs. The infestation by I. ricinus usually occurred in May, while it was least often reported in March and September (Fig. 1).

I. hexagonus species usually invaded in July, and it was least frequently found in March and May (Fig. 2).

In total, the pathogens were found in 26.0% of I. ricinus individuals and in 50.0% of I. hexagonus ticks. This difference was statistically significant (Yates corrected $\chi^2 = 11.23; p = 0.0008$). A. phagocytophilum was found only in 3.4% of female I. ricinus ticks. On the other hand, protozoa of B. microti and T. gondii were reported in both tick species. B. microti was reported in a much higher percentage of I. ricinus and I. hexagonus individuals than T. gondii (Table 1). B. burgdorferi s. l. were not shown in the all study material. In addition, the coexistence of A. phagocytophilum and B. microti, as well as A. phagocytophilum and T. gondii was demonstrated in I. ricinus females (Table 1). In general, all ticks collected from cats and dogs were statistically significantly more often infected with B. microti than with T. gondii (Yates corrected $\chi^2 = 10.96; p = 0.0009$).

Babesia microti was found in 28.1% of the ticks collected from dogs. A. phagocytophilum and T. gondii were found in 3.9% of the I. ricinus females (Table 3). This difference was statistically significant (Yates corrected $\chi^2 = 19.68; p \leq 0.00001$). In addition, single cases of A. phagocytophilum and B. microti as well as A. phagocytophilum and T. gondii were reported in the ticks collected from dogs (Table 3).

As for I. hexagonus ticks collected from cats and dogs, B. microti was shown in 54% of the nymphs and in 33.3% of the females. It should be emphasized that this difference was statistically significant (Yates corrected $\chi^2 = 8.14; p = 0.0043$). T. gondii was demonstrated in 7.1% of the larvae and in 7.7% of the nymphs of this tick species. Generally, hedgehog ticks collected from cats were statistically significantly more often infected with B. microti than with T. gondii (Yates corrected $\chi^2 = 12.79; p = 0.0003$).
Figure 1. The total number of *Ixodes ricinus* ticks collected from domestic animals in Zakopane in 2017–2018.

Figure 2. The total number of *Ixodes hexagonus* ticks collected from domestic animals in Zakopane in 2017–2018.

Table 1. The total number and percentage of *Ixodes ricinus* and *Ixodes hexagonus* ticks infected with *Anaplasma phagocytophilum*, *Babesia microti* and *Toxoplasma gondii* collected from domestic cats and dogs in Zakopane and the surrounding area.
Table 2. The total number and percentage of developmental stages of Ixodes hexagonus ticks infected with Babesia microti and Toxoplasma gondii collected from domestic cats and dogs in Zakopane and the surrounding area.

| Developmental stage | Total number of studied ticks | 1 pathogen | 2 pathogens |
|---------------------|-----------------------------|------------|-------------|
|                     |                             | Babesia microti | Toxoplasma gondii |
| Larva               | 15                          | 7 (46.7%)     | 1 (6.7%)     |
| Nymph               | 13                          | 0 (0.0%)      | 1 (7.7%)     |
| Female              | 8                           | 1 (12.5%)     | 0 (0.0%)     |
| Total               | 36                          | 8 (22.2%)     | 2 (5.5%)     |

Table 3. The number and percentage of Ixodes ricinus females infected with Anaplasma phagocytophilum, Babesia microti and Toxoplasma gondii collected from dogs in Zakopane and the surrounding area.

|                   | Total number of studied ticks | 1 pathogen | 2 pathogens |
|-------------------|------------------------------|------------|-------------|
|                   |                             | Anaplasma phagocytophilum | Babesia microti | Toxoplasma gondii | Anaplasma phagocytophilum + Babesia microti | Anaplasma phagocytophilum + Toxoplasma gondii |
| Females           | 103                          | 4 (3.9%)   | 29 (28.1%)  | 4 (3.9%)     | 1 (1.0%)     | 1 (1.0%)     |
| Total             | 103                          | 4 (3.9%)   | 29 (28.1%)  | 4 (3.9%)     | 1 (1.0%)     | 1 (1.0%)     |

Discussion

Research on the presence of ticks in domestic animals, especially cats and dogs, is carried out around the world, including in Europe. In this study I. ricinus was the predominant tick species infesting domestic dogs and cats, followed by the I. hexagonus. This is in analogy with other studies in Europe8,26–29. The research conducted so far in the areas of southern, south-eastern and central Poland on the occurrence of ticks in domestic cats and dogs confirmed that dogs are usually infested by I. ricinus. Moreover, it has been shown that, in addition to the species mentioned above, other tick species may occasionally infest these animals. These include I. hexagonus, D. reticulatus, I. crenulatus20–22. In Poland, however, studies on the frequency of tick infestation of domestic cats have shown I. ricinus, I. hexagonus, I. rugicollis and Ixodes apronophorus (Schulze, 1924) to be main attackers with I. ricinus dominating in numbers8,23,24. To date, three tick species have been found in Zakopane and the Tatra National Park: I. ricinus, I. hexagonus and I. trianguliceps25. Officially, the first I. hexagonus was collected in the Tatra National Park from the red vole (Myodes glareolus) by Jan Rafalski in 1964. I. trianguliceps is a species associated with rodents. In 1980, however, Hailinger noted a single I. trianguliceps larva feeding on the common weasel (Mustela nivalis)30. Later, an individual of this species was collected from the red vole (Myodes glareolus)31. The studies confirmed the occurrence of I. ricinus and I. hexagonus in Zakopane and the surrounding area and the possibility of infection of domestic cats and dogs. Furthermore, it has been confirmed that I. ricinus is the species most often attacking domestic animals. Selected regions of the Polish Carpathians, including the Island Beskids, are areas of the common occurrence of the tick I. ricinus, recognized in medical and veterinary sense as the most dangerous tick in the Polish fauna of these parasites, and are also the habitat of other tick species, including Carios vespertilionis, Ixodes luidus, Ixodes simplex, Ixodes trianguliceps, Ixodes rugicollis, Ixodes hexagonus, Ixodes persulcatus and other species26,27,29. In the Czech Republic, research was undertaken to check for the occurrence of tick-borne disease pathogens among I. ricinus in mountain areas and it was found that this tick species was recorded even up to a height of 1270 m a. s. l.30. Slovak tick researchers suggest that under the influence of global warming the upper limit of occurrence of these parasites is changing and now I. ricinus ticks can be found even at an altitude of up to 1460 m a.s.l. and I. hexagonus up to 1800 m a. s. l.31. In 2004 and 2006–2011 studies were carried out to check the expansion of ticks to higher altitudes in the ecosystem of Little Fatra (northern Slovakia) and their infection with Babesia microti and Anaplasma phagocyto.

Molecular tests for Babesia microti, Anaplasma phagocytophilum, and Toxoplasma gondii showed that both in ticks species most individuals were infected with Babesia microti. T. gondii was found in a significantly lower percentage of I. ricinus and I. hexagonus ticks. The values obtained are much lower than those received by Asman et al.33 in Tarnowskie Góry County. The researchers showed the presence of B. microti in 42.6% of the individuals, while T. gondii was in 98% of the ticks. In addition, these protozoa have been demonstrated in ticks collected from both cats and dogs33. Contrary to the studies conducted by Asman et al.33, B. microti and T. gondii have been shown in I. ricinus ticks collected only from dogs, and in I. hexagonus species collected only from cats. However, like in the analyses of ticks conducted in Tarnowskie Góry County, both these protozoan species were found mainly in I. ricinus females. This confirms the thesis that, apart from the nymph, this developmental stage is the main epidemiological threat for protozoa32. Ticks may play a large role in the transmission of T. gondii, but this requires further research.

Anaplasma phagocytophilum was found in a much smaller percentage of I. ricinus individuals and was not reported in I. hexagonus. The values are much lower than those obtained by Król et al.3 who demonstrated the
presence of this rickettsia in 21.3% of *I. ricinus* ticks and in 8.1% of *I. hexagonus* individuals collected from cats and dogs in the agglomeration of Wroclaw. Other research conducted in south-eastern Poland also showed a high percentage of *I. ricinus* ticks infected with *A. phagocytophilum*. On the other hand, the values presented in this work are only slightly higher than those presented by Zygnier et al. in central Poland. Similar studies conducted in several European countries on the occurrence of tick-borne pathogens, including *A. phagocytophilum*, in ticks collected from domestic cats and dogs, also showed a higher percentage of *I. ricinus* individuals infected with *A. phagocytophilum*. The studies carried out in the Netherlands revealed a twice lower percentage of *I. ricinus* ticks infected with this rickettsia than in the presented work, while the presence of *A. phagocytophilum* in *I. hexagonus* individuals was significantly higher. There are also cases of *Borrelia burgdorferi* s. l. in ticks collected from domestic animals. The research conducted by Schreiber et al. in Germany showed the presence of this spirochete in 11.6% of *I. ricinus* and in 11.2% of *I. hexagonus* ticks collected from cats and dogs. Studies conducted in The Netherlands and Belgium showed the presence of *B. burgdorferi* s. l. in 7.2% and 10.1% of *I. ricinus* individuals, respectively. Several years of research conducted in urban areas of the Carpathian regions of Slovakia and Poland and their peripheral part showed that specific *Borrelia burgdorferi* s. l. IgG antibodies, were found in 50% of 256 dogs, 6.9% of 29 cats from East Slovakia (Inner West Carpathian) and 42.6% of 68 dogs from the Lublin district. Similar studies carried out in several European countries have shown the presence of this bacterium mainly in *I. ricinus* ticks collected from cats. Studies conducted in France also demonstrated the presence of *B. afzelii* in *I. hexagonus* ticks collected from a cat. The research carried out in Poland has shown that the occurrence of this bacterium in ticks collected from animals may range from 6.2% in *I. ricinus* in central Poland to 22.8% in *D. reticulatus* in south-eastern Poland. The absence of *B. burgdorferi* s.l. in ticks collected from domestic animals in Tatra County may result from the fact that an increase of altitude is related to a decrease in the number of ticks infected with this bacterium, as shown by Taragelova et al. It is commonly known that ticks can be vectors and/or reservoirs of more than one pathogen. There are cases of co-occurrence of two or three pathogens in *I. ricinus* ticks collected from vegetation, but the percentage of such ticks in the population is very low. Also, there are cases of such coinfec tion in ticks collected from domestic animals. The research conducted in Tarnowskie Góry County by Asman et al. showed the co-occurrence of *B. microti* and *T. gondii* in more than 40% of *I. ricinus* ticks collected from cats and dogs. Moreover, co-infection was reported mainly in female *I. ricinus* ticks collected from dogs. On the other hand, Król et al. demonstrated the coexistence of 2 or even 3 pathogens in a single *I. ricinus* tick, with *A. phagocytophilum* and *Rickettsia spp.* most frequently found in co-infection. The ticks collected from dogs in Tatra County showed coexistence of *A. phagocytophilum* and *B. microti* as well as *A. phagocytophilum* and *T. gondii* in only two *I. ricinus* females. However, coexistence of these pathogens was not observed in the ticks collected from cats, which may result from a small number of ticks collected from these animals.

**Conclusions**

The research indicates that potentially unfavourable environmental conditions for ticks in Tatra County do not prevent domestic cats and dogs from a high risk of exposure to the infestation by *I. ricinus* and *I. hexagonus* ticks. In addition, the study revealed a potentially high risk of tick-borne infection of *B. microti* and a low risk of exposure to a tick-borne *A. phagocytophilum* infection and *T. gondii* invasion in the study area. Moreover, the results show possible coexistence of *A. phagocytophilum* and both *B. microti* and *T. gondii* in *I. ricinus* species. However, it cannot be excluded the possibility that some individuals may have been pathogen-positive because of feeding on an infected (asymptomatic) animal. The lack of *Borrelia burgdorferi* s. l. in the material may be due to a generally low percentage of ticks infected with this bacterium in the study area, and this may result from the geographical location.

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Author contributions
K.A.—preparation of the manuscript, collection of research material, identification of tick species and developmental stages, performance of some molecular studies. M.A.—performing some molecular research, assisting in methodology and manuscript discussions. M.N.—performing some molecular research, assisting in methodology and manuscript discussions. M.N.-C.—help in identifying the collected tick species and developmental stages, performance of some molecular studies. M.A.—performing some molecular research, assisting in methodology and manuscript discussions. M.N.—performing some molecular research, assisting in methodology and manuscript discussions. M.N.-C.—help in identifying the collected tick species and developmental stages, performance of some molecular studies.

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Additional information
Correspondence and requests for materials should be addressed to A.K.

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