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THE PREVALENCE OF PIROPLASMS IN A POPULATION OF *IXODES RICINUS* (ACARI: IXODIDAE) FROM NORTH-WESTERN POLAND

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Abstract: Developmental forms of *Babesia* spp. were studied in isolated salivary glands of *Ixodes ricinus*, subjected to the Feulgen reaction. The same ticks were also hosts to *Babesia microti*, which was determined by PCR amplification with primers specific to the fragment of a gene encoding the nuclear small sub-unit ribosomal RNA (SS-rDNA). Presence of *Babesia* spp. was recorded in the salivary glands of 59.9% of ticks collected, both in nymphs and adults. PCR reactions specific to *Babesia microti* were positive in 1.9% of nymphs.

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INTRODUCTION

Piroplasmosis has been posing a significant problem in veterinary parasitology and medical parasitology for the past 30 years. Species of the genus *Babesia* are intracellular parasites of vertebrates. It has been estimated that in endemic areas between 70–100% of cattle are infected with piroplasmosis [13, 24].

The principal aetiological factors of human babesiosis are *Babesia microti* and *B. divergens*. In the USA, over 500 cases of piroplasmosis were caused by on *B. microti* [3, 6, 18, 23], while about 20, mostly fatal, European infections were caused by *B. divergens* [2, 15]. A single Polish case of human babesiosis, most probably imported from Brazil, was caused by *B. microti* [7]. Even though no indigenous babesiosis has yet been recorded in Poland, the persistence of *B. microti* has been reported in a zoonotic reservoir in our country by Karbowiak and Siński [10].

Other favourable factors promoting babesial infections may be the progressive warming of the climate and population increase of *Ixodes ricinus*, known as the principal vector of piroplasms in Europe.

The aim of the present work was to determine the prevalence of the *Babesia* spp., in this number also *Babesia microti* infecting ticks, *Ixodes ricinus* on the forested areas of north-western Poland.

MATERIALS AND METHODS

Nymphs and adult ticks, *Ixodes ricinus* were collected by dragging a 1 m^2 piece of white flannel cloth over low vegetation. From April–October 2001, 8 sites in mixed forest in the vicinity of the city of Szczecin were sampled.

Salivary glands isolated from each specimen, following their fixation in methanol, were subjected to the Feulgen reaction, modified by Piesman [16]. The staining obtained in the course of the reaction enables the differentiation

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between the sporogonic stages of *Babesia* and gland cells of the tick.

B. microti was identified with the aid of polymerase chain reaction (PCR). For the amplification of the *Babesia microti* DNA, we used a fragment of a gene encoding the nuclear small-subunit ribosomal RNA (SS-rDNA) as a target. Primers Bab1 - Bab4 (the expected PCR product is 238 bp in size) described by Persing *et al.* (15) were applied. As a positive amplification control in each reaction, 1 ng of *Babesia microti* merozoite DNA was used. The merozoites were isolated from red blood cells of the infected mice with the aid of a lysing buffer (1:9 mixture of ammonium chloride and Tris buffer). The ammonia method was used to aquire DNA from the merozoites.

Negative amplification control: 5 µl of distilled water added to the PCR buffer was also included in each PCR.

The reactions for detection of *B. microti* consisted of an initial denaturation (1 min at 94ºC) followed by 35 rounds of temperature cycling (94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min). The products of PCR were analysed on 2% agarose gels in TRIS-buffer. Positive results in the form of lanes representing DNA of the size of 238 bp were read in UV using a Smart Ladder transilluminator and Bi o Capt MW computer software developed by Vilbert Lourmat (France).

RESULTS

In total, 461 ticks (405 nymphs, 22 females, and 34 males) were collected. Data in Table 1 indicate that 2 peaks of activity (April and August) occur in the area studied.

Sporozoites of *Babesia* spp. were recovered in almost 60% of the specimens collected (Tab. 1). In June and in October, much higher percentages of infected ticks were recorded, compared to other months. Parasites in the salivary glands were observed in nymphs as well as in adult forms. The latter, both females and males, showed definitely higher prevalence of infection.

Differences in the size and morphology of sporozoites observed indicate their specific diversity (Figs. 2.1, 2.2).

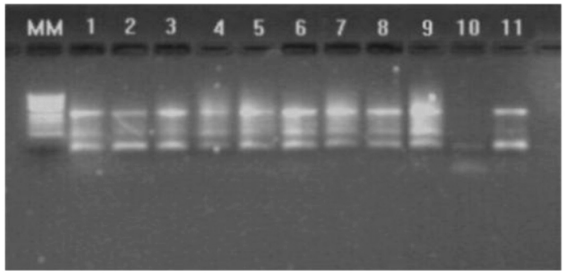

Figure 1. Detection of *B. microti* in *Ixodes ricinus* using polymerase chain reaction. MM- mass marker, 1–9 - positive samples, 10 - negative control, 11- positive control.

Among the fusiform specimens, circular and oval forms were also observed. Light microscope does not enable sporozoite identification up to species level. Hypertrophy of numerous acini with empty sacs, where the parasites were not observed, provides evidence of their earlier presence. Nymphs as well as adults contained sporozoites in primary and secondary acini, which communicated with each other (either directly) or through short ducts, with the main tube of the salivary gland (Fig. 2.3). The presence of sporozoites in tertiary vesicles was observed only in immature specimens. Salivary glands containing large concentrations of sporozoites of *Babesia* spp. demonstrated an advanced hypertrophy and were easily damaged in the procedure of making preparations. In such cases, sporozoites were observed outside the gland cells (Figs. 2.1, 2.2).

Less frequently, salivary gland acini also harboured earlier stages of sporogony: sporoblasts and “fission bodies” (Fig. 2.4).

PCR product of the DNA section specific for *B. microti* was discovered in 1.9% of ticks, which were solely at nymph stage (Fig. 1).

DISCUSSION

To date, very few studies have been carried out on piroplasm infections of ticks. A high percentage of infected ticks in the presently studied population of *Ixodes ricinus*

Table 1. The prevalence of Babesia spp. (Feulgen reaction) and *Babesia microti* (PCR) in a population of *Ixodes ricinus* from north-western Poland.

Month	Number of ticks collected			Percent of ticks infected		
	total	nymphs	male and female	total %F	nymphs %F/%PCR	male and female %F/%PCR
April	103	82	21	57.3	54.9/1.9	66.7/0
May	38	30	8	52.6	50.0/5.3	62.5/0
June	76	68	8	71.1	67.6/2.6	100/0
July	68	60	8	48.5	46.7/2.9	66.7/0
August	95	92	3	65.3	65.2/0	66.7/0
September	38	38	0	44.7	44.7/2.6	0/0
Oktober	43	42	1	72.1	71.4/0	100/0
Total	461	412	49	59.9	58.5/1.9	71.4/0

%F, percentage of ticks infected with *Babesia* spp. (Feulgen reaction); %PCR, percentage of ticks infected with *Babesia microti* (PCR).

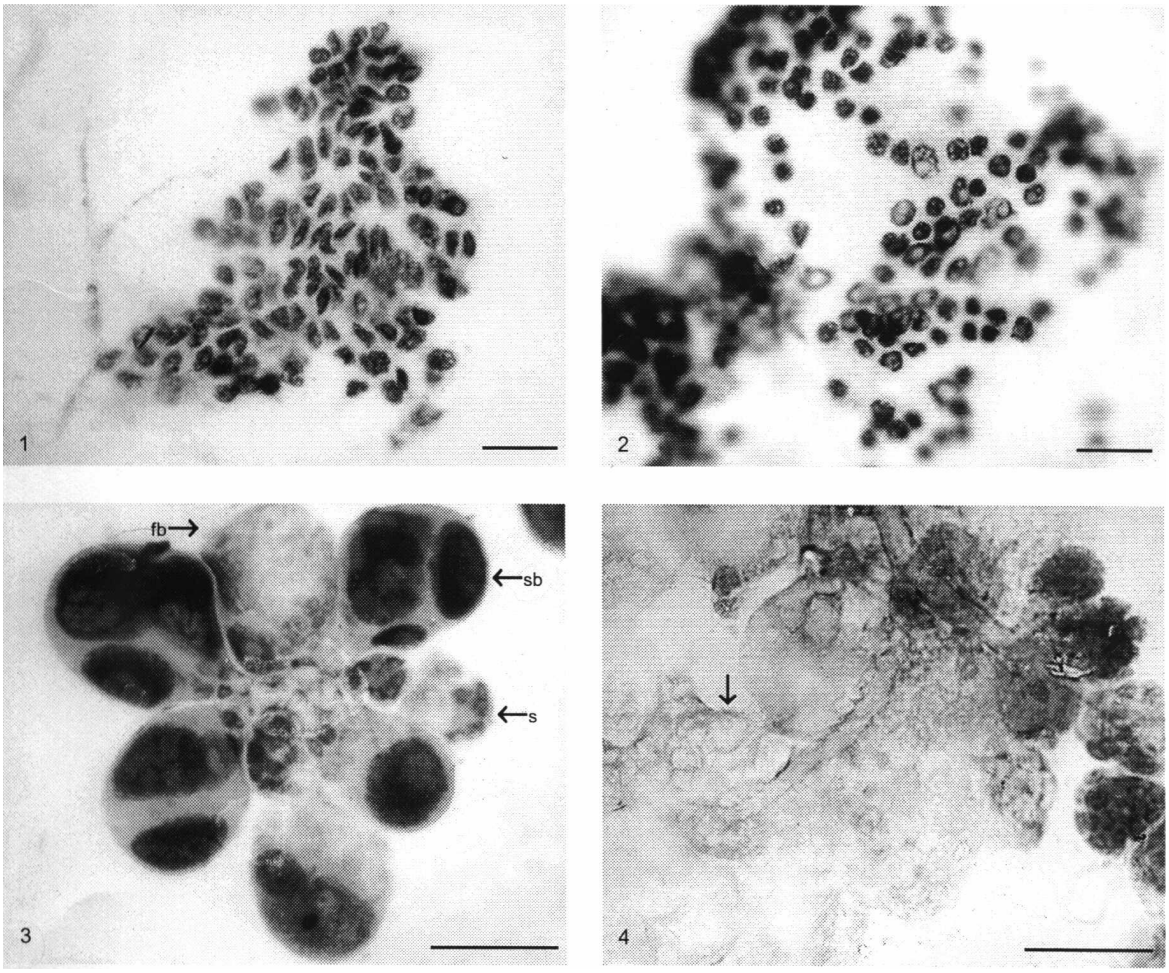

Figure 2. 1) Fusiform sporozoites of *Babesia* sp. from a salivary gland of *Ixodes ricinus*. 2) Oval sporozoites of *Babesia* sp. from a salivary gland of *Ixodes ricinus.* 3) Sporoblasts (sb), fission body (fb), and sporozoites (s) in a salivary gland of *Ixodes ricinus*. 4) Sporozoites of *Babesia* sp. in primary acini of a salivary gland. Empty sacs in hypertrophic acini (arrow). Scale bars: 1) 10 µm; 2) 10 µm; 3) 50 µm; 4) 50 µm.

is consistent with results obtained by numerous authors who have conducted similar studies on different tick species. Akinboade and Dipeolu [1], examining histological preparations of *Boophilus decoloratus*, demonstrated that 61% of nymphs and 46% of adults were infected with *Babesia* spp. In the present work, adults were more infected than nymphs. Because of their higher numbers, nymphs are more important in the epidemiology of transmitted pathogens. It has been demonstrated in the case of *B. microti* that the protozoans survived in only 25% of adult specimens previously infected at nymph stage [16]. The study by Skotarczak and Cichocka [20] on the occurrence of *B. microti* in the population of *I. ricinus* in Poland revealed that infected ticks constituted 13.3%, which substantially exceeded the present results. Extensive differences between our study and the results of other authors can be caused by a number of factors, both ecological and those related to the physiology of ticks.

It is commonly known that transovarian and trans-stadial transmission of *Babesia* contributes to the persistence of high infection rate of ticks with these parasites. No evidence is available, however, on this type of transmission for *B. microti* [10, 19]. This was also evident in the present study where no *B. microti* were present in adult ticks. Low temperature and the time factor limit *B. microti* survival in ticks [17], while longer starvation period of ticks can modify functions of salivary glands and inhibit transmission of piroplasms [8]. The study of Piesman *et al.* [17] demonstrated that after 52 days at 4ºC, the infection rate of nymphs decreased by 50 percent. Based on ultrastructural studies, the above authors concluded that the parasites die in ticks at the sporoblast does not reach the sporozoite stage.

In the present study, *B. microti* were detected by solely in nymphs by using the PCR method.

The small number of ticks infected with this parasite species in the sample taken cannot rule out the possible role of adult forms in the transmission of *B. microti*. Contradictory results on this problem were obtained by scientists who experimentally infected ticks with *B. microti*. Oliveira and Kreier [14], and Walter and Weber [21] observed sporozoites of *B. microti* in the salivary glands of nymphs only, whereas Piesman *et al.* [16] were able to infect also the females.

An additional factor regulating the infection rate of ticks with *Babesia* is the competition with other pathogens transmitted by ticks. Mather *et al.* [11] suggest that *Borrelia burgdorferi* and *Babesia microti* compete within infected ticks, *I. dammini*, because the prevalence of the former is twice as high as the prevalence of the latter. Consequently, in endemic areas there is much higher risk of being infected with Lyme borreliosis than with babesiosis.

The developmental stages of piroplasms in salivary glands of ticks are poorly known. Difficulties in determining the specific identity of the developmental stages of the parasites observed in the salivary glands are further complicated by the different advancement level of sporogony. According to some authors [4, 9, 12], mature sporozoites in the salivary glands are formed during feeding time of ticks. Observations of the present authors, however, indicate that even in many starving specimens, sporozoites were fully developed. Also, multinuclear "fission bodies", known as the earlier sporogony stages, were observed. Structures similar to the "fission body" were observed in *I. ricinus*, experimentally infected with *B. microti* [22] and in the salivary glands of *Haemaphysalis longicornis* infected with *B. gibsoni* [5].

The results of the present study indicate that *Babesia microti* infection of the *Ixodes ricinus* population is low (1.9%) compared to the infection with all *Babesia* species (59.9%).

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