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Parasitological Factors Impeding Transmission of the Babesiosis Pathogen *Babesia microti* from the Tick *Ixodes persulcatus* to Humans

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Abstract—Based on analysis of original and literature data, it is concluded that effective transmission of *B. microti* by the tick *I. persulcatus* is prevented by the following main permanent eco-parasitological factors: lack of pronounced anthropophily in the tick nymphs; low rates of spontaneous invasion of unfed adults; a short duration of the parasitic phase on humans which is not sufficient for completion of sporogony. Therefore, in spite of the possibility of sporadic cases of babesiosis, it can be stated that *B. microti* infection does not and will not play a significant role in infectious pathology within the vast territory where the taiga tick is the only potential source of this infection.

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Babesia microti, the most important agent of human babesiosis from the epidemiological viewpoint, circulates in the natural ecosystems of many countries in the Northern Hemisphere (Tsuji et al., 2001; Gray et al., 2010; Yabsley and Shock, 2013). Its development cycle is well studied; it is realized in erythrocytes of mammals and also in ixodid ticks, in which the schizonts obtained with the blood of infected vertebrates give rise to sporozoites. Only sporozoites can infect intact mammals during the tick's blood feeding, starting the new cycle of babesia development, and can also infect humans (Telford et al., 1993; Telford and Spielman, 1998; Homer et al., 2000). In the United States, where the main vector of these babesias is the deer tick *Ixodes scapularis*, the disease poses a serious problem of infectious pathology. For example, 1124 cases of babesiosis (according to the national case definition) were officially recorded in 2011, with the disease rate in some states being more than 100 per 100 000 population (Herwaldt et al., 2012; Vannier and Krause, 2012). Only sporadic cases of babesiosis of *B. microti* type are recorded in Western and Central Europe, where the main source of human infection is the sheep tick *I. ricinus* (Meer-Scherrer et al., 2004; Gray et al., 2010). So far, no reliable laboratory-confirmed cases of non-imported babesiosis caused by this pathogen have been recorded in Russia.

Considering the role of *I. scapularis* and *I. ricinus* in transmission of *B. microti*, it should be borne in mind that in most cases (about 70%), humans are attacked not by adults but by nymphs of these ticks, which are the main vectors of not only babesial but also Lyme disease infection (Spielman, 1976; Korenberg and Kovalevskii, 1981; Spielman et al., 1985; Piesman et al., 1987; Hubálek et al., 1991; Jaenson, 1991; Telford III et al., 1993; Uspensky, 1993; Stafford et al., 1998; Falco et al., 1999; Gray, 1999; Homer et al., 2000; Robertson et al., 2000; Dennis and Hayes, 2002; Dorn et al., 2002; Hubálek et al., 2004; Nahimana et al., 2004; Wilhelmsson et al., 2013).

The possibility of *B. microti* circulation in the territory of Russia was first demonstrated by detection of the pathogen in the blood of *Myodes* voles from the Middle Ural taiga. The babesia strain obtained from *M. glareolus* proved to be genetically similar to the human pathogen strains from the northeastern United States (GenBank accession number AY094354) (Telford et al., 2002). Later, natural foci of babesiosis were also revealed by molecular biological methods in other regions of Russia (Alekseev et al., 2003; Rar et al., 2011). As in all other cases (Telford III et al., 1993; Homer et al., 2000; Yabsley and Shock, 2013), the reservoir hosts of *B. microti* were several

species of small mammals, of which *Myodes* voles were the most important (Samokhvalov et al., 2010; Rar et al., 2011). The main vector of this babesia is the tick *I. trianguliceps*, which is responsible for its horizontal transmission and dispersal among small mammals (Nefedova et al., 2013) and, as in the western part of its range (Randolph, 1991, 1995; Telford III et al., 1993; Bown et al., 2008), plays the key role in the epizootic chain. This tick is broadly distributed in the forests of Eurasia, from England in the west to the southern shores of Lake Baikal in the east (Korenberg and Lebedeva, 1969). All the active phases of *I. trianguliceps*, namely larvae, nymphs, and adults, parasitize small mammals but do not attack humans (Filippova, 1977); therefore, this tick cannot be the source of any transmissible human infection, including babesiosis. However, in the forest ecosystems in the greatest part of *I. trianguliceps* range, larvae and nymphs of the sheep tick *I. ricinus* and the taiga tick *I. persulcatus*, which definitely participate in the babesiosis epizootic process, occur in great numbers on the same hosts (Bown et al., 2008, Kovalevskii et al., 2013). Of special interest is *I. persulcatus*, whose range covers a vast territory in the forest zone of Eurasia, from the Baltic states to the Russian Far East. The nymphs of *I. persulcatus*, unlike those of *I. ricinus* and especially *I. scapularis* (see above), have a very low level of anthropophily; in the great majority of cases (97–99%) humans are attacked by adult ticks (Fedorov, 1968; Kovalevskii and Korenberg, 1987; Korenberg et al., 1994), which pose the greatest threat as the main vectors of tick-borne encephalitis, ixodid tick-borne borreliosis (ITBB), human granulocytic anaplasmosis (HGA), human monocytic ehrlichiosis (HME), and different variants of mixed infection (Korenberg and Kovalevskii, 1981, 1999; Korenberg et al., 1993, 2013; Korenberg, 1994). It could be expected that within a considerable part of the Eurasian range of *B. microti*, bites of adult taiga ticks carrying this parasite would frequently lead to infection of humans, resulting in considerable incidence of babesiosis.

This study was aimed to analyze the factors preventing the above scenario, and to characterize the epidemic potential of the *B. microti* parasitic systems. For this purpose, we have used material from a certain epizootic territory to determine: (1) whether *B. microti* are present in *I. persulcatus* nymphs which have fed on *Myodes* voles (this being the only way the unfed adult ticks attacking humans could have become carriers of babesias); (2) whether this transmission really takes place, and what is the potential natural level of babesial invasion of unfed adult taiga ticks capable of attacking humans; (3) whether such ticks contain *B. microti* genotypes pathogenic to man; and (4) whether humans can actually be infected with this pathogen.

MATERIALS AND METHODS

The studies were carried out in June–July 2010–2011, at the research station with an area of about 30 km^2 near Mys settlement (58°33′N; 57°28′E) in Chusovskoi District, Perm Territory of Russia. The study area lies in the low-mountain territory of the Middle Urals with the prevalence of southern taiga ecosystems and an almost ubiquitous distribution of the taiga tick *I. persulcatus*. The biocenotic structure of the ecosystems was described previously (Samokhvalov et al., 2010, Kovalevskii et al., 2013; Nefedova et al., 2013). It was in this region of Russia that circulation of *B. microti* was revealed for the first time (Telford et al., 2002).

Nymphs of *I. persulcatus* were collected from small mammals, mostly *Myodes* voles captured with Sherman live traps in forest biotopes. After capture, the voles were kept for several days in individual small cages with a mesh bottom, placed over water-filled trays into which fully engorged ticks could fall. Two drops of blood were taken from the toe of each animal, one on the microscope slide, the other on filter paper. The blood smears fixed and stained after Romanowsky-Giemsa were examined using light field microscopy at 90×7×1.5 magnification. Identification of the observed microorganisms as *B. microti* was then confirmed by PCR testing of the material extracted with PBS from the dry blood samples collected on filter paper. In this way, 32 naturally infected voles were identified, and 46 engorged *I. persulcatus* nymphs were obtained from them and used to prepare suspensions for PCR testing.

Unfed adult *I. persulcatus* ticks were collected from vegetation using the routine flagging technique (Korenberg, 1979), in three areas where their high abundance had been recorded for several years. Altogether, suspensions prepared from 500 mature specimens (349 females and 151 males) were tested for the presence of babesia DNA.

To confirm the clinical and serological diagnoses (Teterin et al., 2013) and reveal the possible cases of babesiosis, we performed PCR screening for the

Table 1. Primers used to amplify microbial DNA from human blood samples and *I. persulcatus* ticks

Primers	Gene	Primer sequences (5'–3')	Size, bp	References
Bab1–Bab4 [1 pair]	Small subunit rRNA (ss-rDNA) of *Babesia microti*	CTTAGTATAAGCTTTTATACAGC ATAGGTCAGAAACTTGAATGATACA	238	Persing et al., 1992
Bab2–Bab3 [nested]		GTTATAGTTTATTTGATGTTCGTTT AAGCCATGCGATTCGCTAAT	154	
IGSb1–IGSa2	Spacer *rrfA-rrlB* of *Borrelia burgdorferi* sensu lato	AGCTCTTATTCGCTGATGGTA CGACCTTCTTCGCCTTAAAGC TATAGGTACCGTCATTATCTTCCCTAT	~260	Derdakova et al., 2003
HE3–MuHE1	16S rRNA gene of *Ehrlichia muris*	TAGTACCCATAGCTTTTTTAGCT ATAGGTTCGC	~400	Anderson et al., 1992; Yu, X.J., pers. comm.
ge3a–ge10r [1 pair]	16S rRNA gene of *Anaplasma phagocytophilum*	CACATGCAAGTCGAACGGATTATTC TTCCGTTAAGAAGGATCTAATCTCC	932	Massung et al., 1998
ge9f–ge2 [nested]		AACGGATTATTCTTTATAGCTTGCT GGCAGTATTAAAAGCAGCTCCAGG	546	

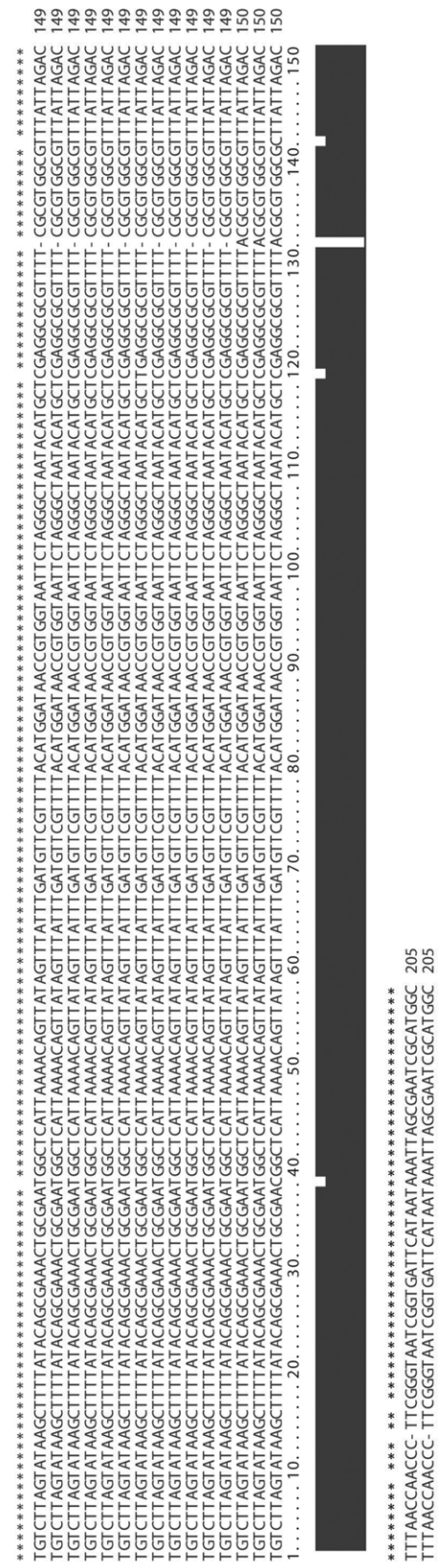

Fig. 1. Alignment of the amplified *B. microti* sequences.

Table 2. Explanations to Fig. 1 (alignment of the amplified *B. microti* sequences)

GenBank accession numbers	Source of material	References	*Babesia* genotype
AY693840	*Homo sapiens*	Slemenda et al., 2004	US type
AY144693	*Clethrionomys* sp.	Goethert and Telford, 2003	US type
HE616519	larva *I. trianguliceps*	Nefedova et al., 2012	US type
KM051827	adult *I. persulcatus*	Nefedova et al., 2014	US type
KM051833	adult *I. persulcatus*	Nefedova et al., 2014	US type
KM051831	adult *I. persulcatus*	Nefedova et al., 2014	US type
KM051832	adult *I. persulcatus*	Nefedova et al., 2014	US type
KM051834	adult *I. persulcatus*	Nefedova et al., 2014	US type
KM051828	adult *I. persulcatus*	Nefedova et al., 2014	US type
KM051830	adult *I. persulcatus*	Nefedova et al., 2014	US type
KM051836	adult *I. persulcatus*	Nefedova et al., 2014	US type
KM051835	adult *I. persulcatus*	Nefedova et al., 2014	US type
AY789075	adult *I. ricinus*	Pieniazek et al., 2006	Munich type
HE616523	larva *I. trianguliceps*	Nefedova et al., 2012	Munich type
KM051829	adult *I. persulcatus*	Nefedova et al., 2014	Munich type

agents of ITBB (*Borrelia burgdorferi* sensu lato), HME (*Ehrlichia muris*), HGA (*Anaplasma phagocytophilum*), and babesiosis in blood samples of 113 out of 583 patients (19.4%) admitted to the First Territorial Isolation Hospital of the city of Perm with acute fever following a tick bite during two spring–summer seasons (2007 and 2010). Blood samples taken upon admission to hospital were examined in 48 patients, those taken 7–14 days after the onset of disease, in 59 patients, and both samples, in six patients.

DNA was isolated from ticks and blood samples using the Proba-NK commercial set (DNA-Technology LLC, Moscow, Russia). Amplification was carried out in a Tertsik four-channel thermocycler (DNA-Technology LLC), with previously suggested primers flanking certain bacterial gene fragments (Table 1). The primers were synthesized by the amidophosphite method at Syntol LLC (Moscow, Russia). The DNA of *A. phagocytophilum* and *B. microti* was amplified by the nested PCR method. The DNA of the following microorganisms was used as positive controls: *B. afzelii* type strain Ip-21, *B. garinii* isolate Ir-2200 (from the collections of the Laboratory of Infection Vectors, N.F. Gamaleya Research Institute for Epidemiology and Microbiology, Moscow), *E. muris*, *A. phagocytophilum*, and *B. microti* (from the corresponding corpuscular antigens for IIF, kindly provided by Dr M. Levin, CDC, United States). The amplicons were resolved by horizontal electrophoresis at 165 V in 1–2% agarose gel with ethidium bromide and tris-borate buffer and analyzed using the DNA Analyzer video system and Gel Imager and Gel Analysis v. 1.0 software.

Fragments of the *B. microti* ss-rDNA gene (238 bp) from 11 DNA samples obtained from unfed adult *I. persulcatus* ticks were sequenced using an ABI PRISM® BigDye™ Terminator v. 3.1 set, and the products were analyzed on an Applied Biosystems 3730 DNA Analyzer at the GENOME Collective Access Center (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow; http://www.genome-centre.ru). The sequences were identified in GenBank/EMBL/DDBJ databases using the BLASTN tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Comparison and analysis of these nucleotide sequences were performed using the MEGA v. 3.1 software package. The sequences of 10 fragments of ss-rDNA gene of *B. microti* were deposited in GenBank/EMBL/DDBJ under accession numbers KM051827–KM051836.

RESULTS

The presence of *B. microti* DNA was revealed in 12 out of 46 (26.1 ± 12.9%) *I. persulcatus* nymphs that had fed on *Myodes* voles naturally infected by these

babesias. Among the 500 tested unfed adult taiga ticks, positive results of PCR with species-specific primers were obtained in only 15 cases (3.0 ± 1.5%).

The amplicons obtained from 10 out of the 15 PCR-positive ticks were sequenced. Figure 1 and Table 2 show the results of their analysis in comparison with the corresponding sequences of babesias from the reservoir hosts and various vectors, available from GenBank. In nine cases, the sequences amplified from unfed adult *I. persulcatus* were totally identical to those of the pathogenic US-type *B. microti* isolated from a patient (GenBank AY693840), reservoir hosts (AY144693), and the main vector of these babesias, the tick *I. trianguliceps* (HE616519) from the natural focus under study. One of our sequences (KM051829) was almost completely identical to the sequences of the non-pathogenic Munich-type *B. microti* isolated from *I. ricinus* in Western Europe (AY789075) and from *I. trianguliceps* in our study region (HE616523).

PCR testing of 113 human blood samples revealed the DNA of *B. burgdorferi* sensu lato in 84 cases; markers of the HME pathogen, in 13 cases, those of HGA, in nine cases; and *B. microti*, in two cases. PCR of blood samples from five patients with primers for these agents yielded no positive results; these patients were diagnosed with tick-borne encephalitis by their attending physicians, based on the clinical and serological data. The PCR results generally agreed with the established diagnoses (Teterin et al., 2013), except for the fact that both patients who tested positive for *B. microti* DNA did not show any characteristic clinical signs of babesiosis; they had an enterovirus infection.

DISCUSSION

We do not intend to present one more review of the etiologically and epidemiologically diverse group of babesia-related diseases, or to repeat the detailed descriptions of the babesia development cycle and its relations with the reservoir hosts and vectors (Telford et al., 1993; Telford and Spielman, 1998; Homer et al., 2000; Vasilieva et al., 2008; Gray et al., 2010; Vannier and Krause, 2012; Yabsley and Shock, 2013). Instead, let us consider to what extent the specific results of our research shed light on the main topic of this communication. It should be noted that detection of *B. microti* DNA in unfed *I. persulcatus* adults is not necessarily equivalent to the presence of the living pathogen itself; therefore, we can only estimate the potential role of this tick in babesiosis epidemiology.

Unfed adult taiga ticks can obtain *B. microti* kinetes only by transstadial transmission from nymphs. In turn, the nymphs can be naturally infected by feeding on small mammals or by transmission from larvae. Formation of babesia gametes from gametocytes starts in the tick larvae within several minutes after the beginning of feeding on the infected mammal. It was experimentally shown for *I. scapularis* (Karakashian et al., 1983; Rudzinska et al., 1984) that babesia gametogenesis ended even before complete engorgement of the ticks. In any event, of crucial significance is the level of invasion of the tick nymphs after their feeding on the reservoir hosts, regardless of the way (vertical or horizontal) of their initial infection. Our results show that babesia DNA can be detected in approximately one-fourth of the *I. persulcatus* nymphs that have fed on the naturally infected red-backed voles. In the natural ecosystem where the babesias-infected voles were captured, the preimaginal phases of the taiga tick and all the phases of *I. trianguliceps* (the main vector of babesias) simultaneously occur on the hosts of the same species and even of the same demographic groups. Moreover, these ticks have a tendency for simultaneous feeding on certain host individuals. For example, according to the long-term data, both species of ticks were found on 68% of the examined *M. rutilus* voles in June, and on 24% of these voles in August (Kovalevskii et al., 2013). This phenomenon certainly facilitates babesia infection of preimaginal phases, in particular nymphs of *I. persuulcatus*. At the same time, it should be borne in mind that ticks in nature attack not only the voles infected with babesias, which appear to comprise about half among *Myodes* voles of three species in the studied ecosystem (Samokhvalov et al., 2010), but also non-infected small mammals. In addition, a considerable proportion of *I. persulcatus* nymphs may feed on larger mammals and on birds (Korenberg et al., 2013), which are not involved at all in the circulation of *B. microti*. Therefore, our somewhat "artificial" estimate of the infection rate of *I. persulcatus* nymphs may be higher than their actual spontaneous infection rate in the natural foci.

Among the unfed adult taiga ticks in our material, babesia DNA was detected in only a small proportion of specimens, no more than several percent. Taking

into account the statistical error, this proportion was 3 to 26 times smaller than that of the nymphs which have fed on the infected reservoir hosts. Fairly similar results of PCR testing of unfed mature taiga ticks were obtained in other regions of Russia, and also in Japan (Alekseev et al., 2003; Rar et al., 2010, 2011; Zamoto-Niikura et al., 2012). One of the possible causes of this situation was considered above. One more cause may be the relatively low frequency of transstadial *B. microti* transmission of from nymphs to adults of *I. persulcatus*, though there are no corresponding data in the literature. The low level of infection (several percent) of adult ticks attacking humans appears to be an important factor determining the rarity or even absence of cases of babesiosis in the territory where the taiga tick is the only potential vector of *B. microti*. It should be noted for comparison that at the current intensity of human contacts with natural foci of tick-borne encephalitis, a similar level of tick infection with the virus determines a high level of epidemic manifestation of the disease (Korenberg et al., 2013), including Perm Territory where our research was conducted (Korenberg et al., 2001). This may be explained by generalization of the viral infection in the ticks: at the moment of attack, the agent is already present in the tick's salivary glands and is inoculated into human host with the first portion of saliva. In other words, infection in most cases occurs already before the attached tick is removed (see below). The same is true of borreliae (Alekseev, 1993; Korenberg et al. 2013).

Several genotypes of *B. microti* are currently known (Tsuji et al., 2001; Gray et al., 2010; Ohmori et al., 2011; Zamoto-Niikura et al., 2012). Two of them are the most widespread in Eurasia: the US type that causes human babesiosis, and the "Munich" type whose significance as a human pathogen remains unproven. Both of them were revealed in the ecosystem where our previous research was conducted (Telford et al., 2002; Nefedova et al., 2013). In the new material, we detected the DNA of Munich-type *B. microti* not only in the grey red-backed vole *M. rufocanus* and in the larva of the tick *I. trianguliceps*, but also in the unfed adult tick *I. persulcatus*. This finding confirmed our previous conclusion that at least two genotypes of *B. microti* could simultaneously circulate in the biocenosis using the same reservoir hosts and vectors (Nefedova et al., 2013). In most cases, however, the taiga ticks revealed the markers of the pathogenic US type. Thus, our data demonstrate the absence of any microbiological or etiological factor that can prevent human infection with babesias from adult *I. persulcatus* ticks.

In addition to the low level of infection of adult taiga ticks, their limited role as a source of human infection may be explained by one more essential parasitological trait related to the development of babesias. The only invasive stage of *B. microti* that can be transferred to the vertebrate host is that of sporozoites. Sporogony, i.e., formation of sporozoites, takes place in the tick's salivary glands after the beginning of blood feeding and takes some time. The tick becomes capable of transferring babesias to man not earlier than two days after its attachment and the beginning of feeding (Piesman and Spielman, 1980, 1983; Karakashian et al., 1983). However, representative data show that adult *I. persulcatus* ticks get removed within the first two days after attachment in 90.9–97.6% of the cases, and within the first day in 88.5–93.6% of the cases (Yarotsky, 1960; Korenberg et al., 1994), i.e., before the sporogony of babesia is completed. These facts confirm the opinion of Balashov (2005, 2009) that the low incidence of human infection may be accounted for by the removal of ticks at early stages of their feeding, before the appearance of the invasive stages of babesias. This explanation appears to be perfectly valid in the case of *B. microti* and *I. persulcatus*. It is significant that a noticeable number of clinically manifested cases of this babesiosis was recorded in those regions where humans are attacked not only and not so much by adult *I. scapularis* and *I. ricinus* ticks as by their less conspicuous nymphs, unlike in case of *I. persulcatus*. In about half of the cases, ticks of these two species remain on the bodies of their victims for more than 24 hours (up to 96 hours or even longer) before being removed (Falco et al., 1996; Wilhelmsson et al., 2013). Still, as noted above, even though nymphs of *I. ricinus* often attack humans, the incidence of babesiosis caused by *B. microti* within the range of this tick is much lower than could be expected. A possible explanation of this phenomenon is that since the babesia types circulating in Europe are weakly pathogenic to man, the mild form of disease caused by them may be clinically underdiagnosed (Kjemtrup and Conrad, 2000; Meer-Scherrer et al., 2004). This assumption is supported by a considerable proportion of positive serological tests among humans bitten by ticks (Hunfeld and Brade, 2004). The microbiological and clinical laboratory data related to this problem are still very

scarce. The factors determining the low incidence of diseases caused by *B. microti* within the range of *I. ricinus* require further research, and the diagnostics of babesioses should certainly be based on the modern serological and molecular methods of pathogen detection. It cannot be excluded that *B. microti* transmitted by the taiga tick may also cause mild forms of disease with indistinct clinical signs, depending not only on the pathogenicity of particular types (genotypes, genovariants) of the agent but also on individual susceptibility (the premorbid status) of the host organism. It is significant in this connection that early serological analysis of patients who developed diseases of unknown etiology after the attacks of *I. persulcatus* ticks in the study region, yielded negative results with the antigens of both Russian and American pathogenic strains of *B. microti* (Telford et al., 2002). The results of more sensitive genetic testing for borreliosis were also negative.

Thus, analysis of our own and published data allows us to conclude that effective transmission of *B. microti* by the taiga tick *I. persulcatus* is prevented by the following main eco-parasitological factors, which are permanent and act at the system level: the lack of pronounced anthropophily in the tick nymphs; a low level of spontaneous infection of unfed adult ticks; quick removal of the attacking ticks, which remain on the human host for too short a period for sporogony to be completed. Therefore, it may be stated that, even though sporadic cases of babesiosis are possible in the vast territory where the taiga tick is the only potential source of babesial infection, this parasite is not and will not be important from the standpoint of infectious pathology.

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