



The first study of the prevalence and genetic diversity of *Theileria equi* and *Babesia caballi* in horses in Russia

Vera Rar¹ · Victor Marchenko² · Olga Suntsova³ · Tamara Epikhina¹ · Artem Tikunov¹ · Ivan Meltsov⁴ · Valeria Fedorets¹ · Yana Igolkina¹ · Irina Kozlova³ · Nina Tikunova¹

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Abstract

Equine piroplasmosis (EP) is a global worldwide infection, which can lead to the death of animals. Despite the causative agents of EP being well studied, there are no data on the distribution and genetic characteristics of EP agents in any region of Russia. In this study, blood samples from 750 horses from Novosibirsk province, Irkutsk province, and Altai region of Russian Siberia were examined for the presence of EP agents. *Theileria equi* and *Babesia caballi* were detected in all examined regions, with mean prevalence rates of 60.4% and 7.2%, respectively. The identified pathogens were genetically characterized by the 18S rRNA gene. The determined *T. equi* sequences were highly conserved and belonged to genotypes A and E, with genotype E being found in 88.6% of genotyped samples. In contrast to *T. equi*, *B. caballi* sequences were genetically diverse. Seven sequence variants of *B. caballi* were identified, and only two of them matched known sequences from the GenBank database. The determined *B. caballi* sequences belonged to four distinct branches within genotype A. Mixed infections with several variants of *B. caballi* or with *T. equi* and *B. caballi* were common. The conducted phylogenetic analysis based on all available *B. caballi* sequences of the 18S rRNA gene (> 900 bp) from GenBank and from this study first demonstrated the presence of five monophyletic clusters within genotype A and three clusters within genotype B. Thus, the genetic study of *B. caballi* from Siberia has significantly expanded the data on the genetic diversity of this pathogen.

Keywords *Theileria equi* · *Babesia caballi* · Equine piroplasmosis · Phylogenetic analysis · New genovariants · Russian Siberia

Introduction

Theileria equi [Theileria, Theileriidae, Piroplasmida, Apicomplexa] and *Babesia caballi* [Babesia, Babesiidae, Piroplasmida, Apicomplexa] are the main causative agents of

equine piroplasmosis (EP) worldwide (Tirosh-Levy et al. 2020a). Recently, a new species, *T. haneyi*, closely related to *T. equi*, has been discovered in equine blood based on complete genome sequencing (Knowles et al. 2018). EP is found mainly in Equidae—horses, donkeys, mules, zebras. Reported cases of infections in other animals are rare (de Sousa et al. 2018; Onyiche et al. 2019; Qablan et al. 2013).

The life cycles of both *Theileria* and *Babesia* include asexual reproduction (merogony) in the erythrocytes of equine hosts and sexual (gamogony) and asexual (sporogony) stages within the ticks; however, they have their own peculiarities. Thus, *Theileria* spp., but not *B. caballi*, undergoes asexual schizogony within equine lymphocytes before the penetration into red blood cells. Another difference is that only *B. caballi* can be effectively transmitted transovarially to the next generation of ticks (Scoles and Ueti 2015; Wise et al. 2013).

Equine piroplasmosis has emerged as a global worldwide infection in the beginning of twentieth century. The infection

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✉ Vera Rar
rarv@niboch.nsc.ru

- ¹ Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russia
- ² Federal Altai Scientific Center for Agrobiotechnology, Barnaul, Russia
- ³ Scientific Centre for Family Health and Human Reproduction Problems, Irkutsk, Russia
- ⁴ Irkutsk State Agrarian University Named After A.A. Ezhevsky, Irkutsk Province, Molodezhny Settlement, Russia

first emerged in Southern Africa and then spread to other continents, including Eurasia. The disease posed a serious threat to the economy due to high morbidity and mortality rates in horses. EP is a reportable infection by the World Organization for Animal Health (WOAH) (<https://www.woah.org/>). Today, only several countries report EP-free status, namely, Australia, Canada, Great Britain, Ireland, Japan, New Zealand, and, until a large outbreak in 2009, the United States (Tirosh-Levy et al. 2020a).

Equine piroplasmiasis can occur in acute, subacute, and chronic forms. Acute infection is characterized by fever up to 40 °C, loss of appetite, weakness, anorexia, mucosal edema, splenomegaly, thrombocytopenia, and hemolytic anemia leading to hemoglobinuria and jaundice. In some cases, infection can lead to abortions (Dirks et al. 2021; Wise et al. 2013). Both parasites cause similar pathology, but clinical presentations are more severe in cases of *T. equi* infection (Tirosh-Levy et al. 2020a; Wise et al. 2013). Horses that recovered from an acute infection remain persistently infected by pathogens without any clinical manifestations; however, they can transmit protozoan pathogens to tick vectors. Typically, the persistence of *T. equi* is lifelong, while *B. caballi* is cleared within several years (Scoles and Ueti 2015; Wise et al. 2013). The level of long-termed parasitemia is usually low, so EP can be diagnosed mainly by serological or molecular methods, but not by pathogen finding in blood smears (Scoles and Ueti 2015). Expectedly, clinical cases of both *T. equi* and *B. caballi* are associated with higher mean parasite load compared to subclinically infected horses (Tirosh-Levy et al. 2020b). In endemic areas, the proportion of infected animals is often high, exceeding 60% (Bhoora et al. 2020; Dahmana et al. 2019; Díaz-Sánchez et al. 2018). *Theileria equi* is a more common agent than *B. caballi*. In 20-year period (2000–2019), the global estimated prevalence of *T. equi* and *B. caballi* infections was 34.6% and 7.4%, respectively, being the highest in South America (Tirosh-Levy et al. 2020a).

The natural foci of EP cannot be maintained in the absence of competent vectors. At least 33 ixodid species, mainly belonging to the genera *Hyalomma*, *Dermacentor*, and *Rhipicephalus*, have been implicated as competent vectors for both *B. caballi* and *T. equi* (Scoles and Ueti 2015; Onyiche et al. 2019).

Nowadays, the danger of EP is less significant because of a lower abundance of horses, the availability of modern acaricid and antiprotozoal drugs, and a wide distribution of pre-immune horse population. Cases of acute infection in endemic areas are rare; young animals are exposed to infected ticks when they are usually protected by maternal antibodies, and exposure to infection can lead to the development of strong immunity. Outbreaks primarily occur when naive horses are transported into endemic areas or when infected animals are imported into non-endemic areas where

competent tick vectors are abundant (Scoles and Ueti 2015). Therefore, the outbreaks of EP periodically occur in both endemic and non-endemic regions from various countries (Fedulina et al. 2014; Ionita et al. 2018; Short et al. 2012; Tirosh-Levy et al. 2020b).

Significant genetic variability was shown for both *B. caballi* and *T. equi*. Based on the study of the 18S rRNA gene, three genotypes of *B. caballi* (A, B1, and B2) and five genotypes of *T. equi* (A–E) have been identified (Bhoora et al. 2009, 2020; Tirosh-Levy et al. 2020a); however, in 2018, some *T. equi* isolates of genotype C were assigned to a new species, *T. haneyi* (Knowles et al. 2018). Distribution of different *T. equi* genotypes and *T. haneyi* depends on geography (Tirosh-Levy et al. 2020a). Notably, *T. equi* and *T. haneyi* cannot be distinguished by the 18S rRNA gene phylogeny but only based on the presence of equi merozoite antigen 1 (ema-1) gene in *T. equi* and hypothetical protein gene of unknown function in *T. haneyi* isolates (Knowles et al. 2018).

Mixed infection with these three EP agents as well as simultaneous infection with several *T. equi* genotypes has been described in many studies (Bhoora et al. 2020; Coultous et al. 2020; Elsayy et al. 2021). Moreover, in some regions, infection of equines with multiple *T. equi* genotypes prevailed over single genotype infection (Coultous et al. 2020). There is no reliable evidence that a specific *T. equi* genotype correlates with increased virulence; however, two studies demonstrated a higher frequency of genotype A in clinically affected horses compared with genotype D (Tirosh-Levy et al. 2020b) and genotype B (Manna et al. 2018).

In Russia, EP was first recorded in 1906–1908 in the Ryazan province; subsequently, the infection was found in various provinces of Russia. In the 1930–1950s, the epizootic situation regarding EP significantly worsened due to the movement of a large number of horses to endemic areas; there was a massive loss of animals. This was the reason for conducting large-scale studies of this disease in the USSR. Both pathogens were identified in the blood of horses from endemic areas and the role of *Hyalomma scupense*, *Rhipicephalus bursa*, *Dermacentor silvarum*, *Dermacentor nuttalli*, and *Dermacentor marginatus* as carriers of these hemoparasites was proved (Abramov 1955; Budnik 1955; Ovchinnikov et al. 1941; Petunin 1948; Scoles and Ueti 2015). In the 1960s, the EP incidence in Russia decreased significantly. However, cases of EP are periodically observed in various regions of Siberia. In particular, in 2008, an outbreak of infection was noted in the Ust-Udinsky district of the Irkutsk province (Eastern Siberia), which led to the death of animals (Fedulina et al. 2014). Unfortunately, EP has not been practically studied in Russia in recent decades. There are only fragmentary data on cases of this disease, and the diagnosis in most

cases was based only on the symptoms. Moreover, there are no data on the genetic diversity of the causative agent of EP from any regions of Russia.

This study was aimed to investigate the epidemiology of EP in three remote regions of Western and Eastern Siberia and to examine genetic variability of revealed EP agents.

Materials and methods

Sampling

A total of 750 blood samples were collected in 2012–2022 from apparently healthy horses from randomly chosen farms, stables, and equestrian centers from two districts of Novosibirsk province ($n = 20$), 13 districts of Irkutsk province ($n = 424$), and eight districts of Altai region, including seven districts of the Republic of Altai ($n = 286$) and one district of the Altai Territory ($n = 20$)

(Fig. 1). Blood samples (5 ml) were taken aseptically from each animal using VACUETTE® tubes containing EDTA (Greiner Bio-One, Austria), transported to the laboratory and stored at $-20\text{ }^{\circ}\text{C}$ until DNA extraction.

Molecular genetic analysis

Total DNA was extracted from 200 μl of each blood sample using the Proba NK kit (DNA-Technology, Moscow, Russia) according to the manufacturer's protocol. Piroplasmida DNA was detected by nested PCR for 18S rRNA gene using primer sets BS1/BS2 and PiroA/PiroC. For species determination, additional nested reactions were performed independently using primers Teq1 and Teq2, specific to *T. equi*, and primers Bcab1 and Bcab2, specific to *B. caballi* (Table 1). All PCR reactions were conducted under conditions previously described in Rar et al. (2011). The annealing temperature and the length of obtained PCR fragments are specified in Table 1. The amplified 18S rRNA gene fragments of all *B. caballi* and some *T. equi* specimens were sequenced. For subsequent sequencing of longer fragments, 1218–1252 bp

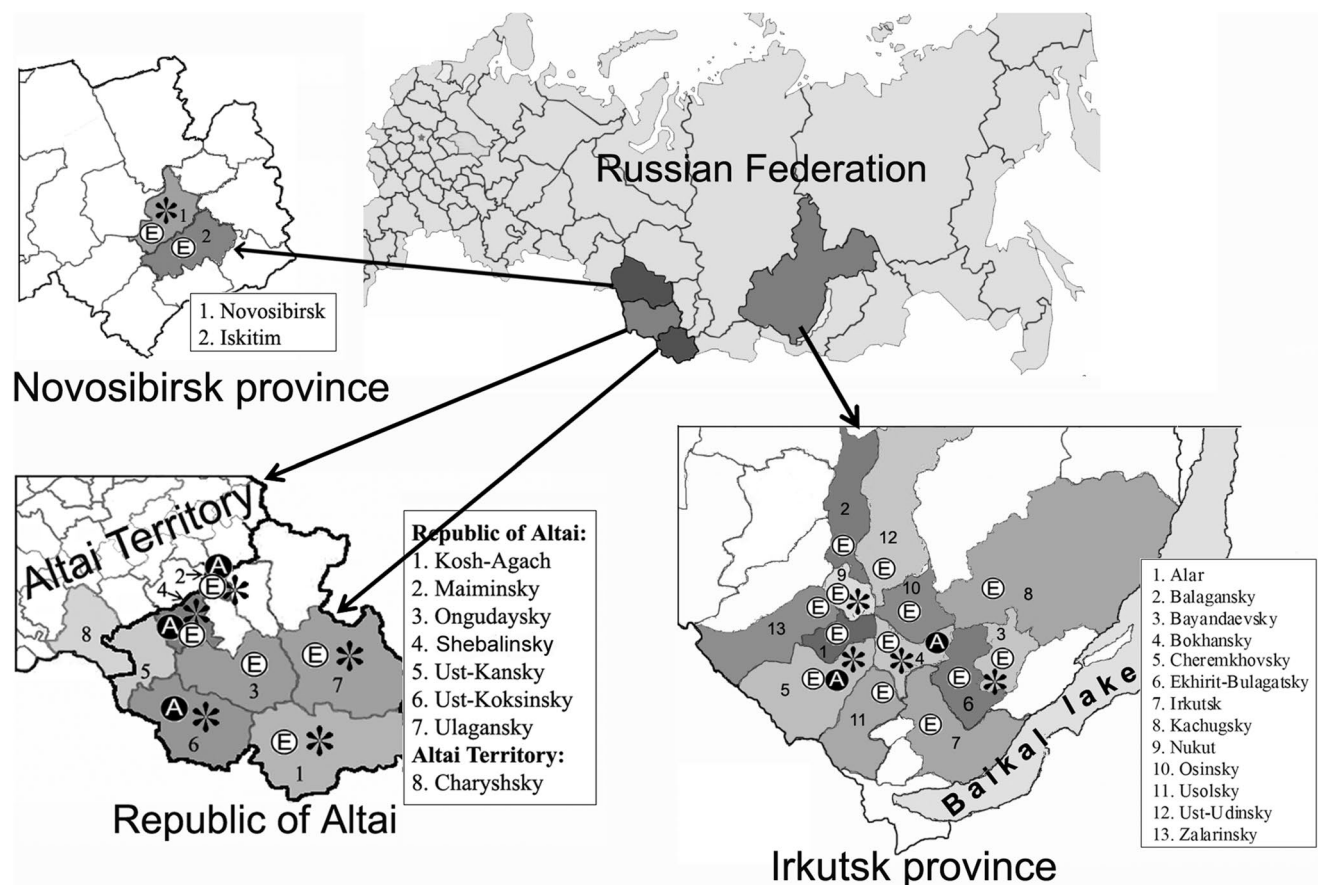


Fig. 1 Map of three regions of Russian Siberia showing the sites of sampling. The districts where horse blood samples were collected are marked by dark color. The districts where EP agents were identified

were marked by \textcircled{E} —*T. equi* genotype E, \textcircled{A} —*T. equi* genotype A, * —*B. caballi*

Table 1 Primers used for detection and genotyping of *Theileria equi* and *Babesia caballi*

Organism/genetic locus	Reaction	Primer name	Primer sequences (5'-3')	T* (°C)	PCR fragments (bp)	References
Piroplasmida/18S rRNA	Primary	BS1	gacggtagggtattggcct	58	1290–1322	Rar et al. 2011
		BS2	attcaccggatcactcgatc			
Piroplasmida/18S rRNA	Nested	PiroA	attaccaatctctgacacaggg	64	342–371	Rar et al. 2011
		PiroC	ccaacaaaatagaaccaargtcctac			
<i>T. equi</i> /18S rRNA	Nested	Teq1	gggaatttaaacccctccag	55	869–870	This study
		Teq2	tccttgcgatttatgaccgc			
<i>B. caballi</i> /18S rRNA	Nested	Bcab1	gatggcgacttaaacctcgc	55	791–792	This study
		Bcab2	aaaagggaagctagttagcag			
<i>Theileria</i> spp./18S rRNA	Nested	BS3	taccgggacgacgacgggtg	60	1251–1252	Rar et al. 2011
		BS4	agggacgtagtggcaccag			
<i>Babesia</i> s.s./18S rRNA	Nested	BS5	cgaggcagcaacgggtaacg	60	1218–1219	Rar et al. 2011
		BS6	agggacgtaatctgcacaag			

* Annealing temperature

fragments were amplified for a number of *T. equi* and *B. caballi* specimens using primer sets Bs3/Bs4 and Bs5/Bs6, respectively (Table 1).

The obtained PCR products were gel purified (0.6% SeaKem® GTG-agarose, Lonza, Haifa, Israel) and sequenced. Sanger reactions were conducted using the Big-Dye Terminator V. 3.1 Cycling Sequencing Kit (Applied Biosystems, CA, USA) and visualized with a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For sequence analysis, BlastN (<http://www.ncbi.nlm.nih.gov/BLAST>) and BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) programs were used. Phylogenetic trees were constructed using the maximum likelihood (ML) method based on the Tamura-Nei model in MEGA 7.0 with 1000 bootstrap replicates (Kumar et al. 2016) based on sequences from this study and sequences from the GenBank database available on 1 March 2024. The stability of the constructed trees was estimated by bootstrap analysis with 1000 replicates.

Differences in the prevalence of infectious agents were computed using the Pearson χ^2 goodness-of-fit test (<http://www.socscistatistics.com/tests/chisquare/>). $P < 0.05$ was regarded as significant.

Results

Theileria equi and *B. caballi* prevalence in equine blood

Among 750 examined equine blood samples, 468 (62.4%) samples contained Piroplasmida DNA. In different regions, the portion of infected horses was similar and varied from 55.6% in Altai region to 67.5% in Irkutsk province (Table 2). However, the proportion of infected horses varied

significantly between districts within the region. In Altai region, the prevalence of EP agents ranged from 0% (Ust-Kansky districts of the Republic of Altai and Charyshsky district of the Altai Territory) to 100% (Kosh-Agach districts of the Republic of Altai). In Irkutsk province, the portion of infected horses varied from 20% (Osinsky district) to 97.0% (Nukut district) (Table 2).

Theileria equi and *B. caballi* identification was carried out for all positive blood samples using species-specific PCR. Both EP agents were detected in all regions, with *T. equi* prevalence being significantly higher ($P < 0.01$) compared to *B. caballi* prevalence. Including cases of mixed infection, *T. equi* was detected in 55.0%, 66.3%, and 52.6% of horse blood samples collected in Novosibirsk province, Irkutsk province, and Altai region, respectively; the prevalence of *B. caballi* in different regions ranged from 5.0 to 10.1%. Notably, *B. caballi* significantly more frequently ($P < 0.01$) was found simultaneously with *T. equi* (5.2%) than as a mono-infection (2.0%) (Table 2).

Theileria equi genetic variability

Among 453 *T. equi* samples identified in equine blood, 281 samples were genetically characterized by the 18S rRNA gene. For all genotyped samples, 400–750 bp fragments were sequenced, including the V4 hypervariable region. In addition, 1190–1195 bp fragments were sequenced for 30 samples from different locations. Based on conducted phylogenetic analysis, all determined *T. equi* sequences belonged to two clusters corresponding to genotypes A and E (Fig. 2). Genotype E prevailed in all regions, being found in all genotyped samples from Novosibirsk province, 84.1% (122/145) samples from Irkutsk province, and 92.9% (118/127) samples from Altai region (Table 3). Moreover, genotype E had

Table 2 Detection of Piroplasmida DNA in equine blood samples

Region	District	No. of samples	No. (%) of samples containing DNA				
			All Piroplasmida	<i>T. equi</i>	<i>B. caballi</i>	<i>T. equi</i> + <i>B. caballi</i>	
Novosibirsk province	Iskitim	5	4 (80.0)	4 (80.0)	0	0	
	Novosibirsk	15	8 (53.3)	7 (46.7)	1 (6.7)	0	
	All districts	20	12 (60.0)	11 (55.0)	1 (5.0)	0	
Irkutsk province	Alar	45	40 (88.9)	39 (86.7)	0	1 (2.2)	
	Balagansky	30	24 (80.0)	23 (76.7)	0	1 (3.3)	
	Bayandaevsky	20	11 (55.0)	6 (30.0)	1 (5.0)	4 (20.0)	
	Bokhansky	30	17 (56.7)	12 (40.0)	4 (13.3)	1 (3.3)	
	Cheremkhovskiy	66	57 (86.4)	57 (86.4)	0	0	
	Ekhirit-Bulagatsky	20	16 (80.0)	12 (60.0)	0	4 (20.0)	
	Irkutsk	38	21 (55.3)	21 (55.3)	0	0	
	Kachugsky	20	14 (70.0)	14 (70.0)	0	0	
	Nukut	35	34 (97.1)	28 (80.0)	0	6	
	Osinsky	20	5 (20.0)	5 (20.0)	0	0	
	Usolsky	30	10 (33.3)	10 (33.3)	0	0	
	Ust-Udinsky	40	14 (35.0)	14 (35.0)	0	0	
	Zalarinsky	30	23 (76.7)	23 (76.7)	0	0	
	All districts	424	286 (67.5)	264 (62.3)	5 (1.2)	17 (4.0)	
Altai region	Kosh-Agach*	58	58 (100)	54 (93.1)	0	4 (6.9)	
	Maiminsky*	33	11 (33.3)	9 (27.3)	2 (6.1)	0	
	Ongudaysky*	17	9 (52.9)	9 (52.9)	0	0	
	Shebalinsky*	71	32 (45.1)	18 (25.4)	5 (7.0)	9 (12.7)	
	Ust-Kansky*	23	0	–	–	–	
	Ust-Koksinsky*	15	5 (33.3)	3 (20.0)	2 (13.3)	0	
	Ulagansky*	69	55 (79.7)	46 (66.7)	0	9 (13.0)	
	Charyshsky [#]	20	0	–	–	–	
		All districts	306	170 (55.6)	139 (45.4)	9 (2.9)	22 (7.2)
	All regions	2012–2022	750	468 (62.4)	414 (55.2)	15 (2.0)	39 (5.2)

*Districts from the Altai Republic

[#]A district from the Altai Territory

a wider distribution compared to genotype A; it was identified in two districts in Novosibirsk province, 13 districts in Irkutsk province, and five districts in Altai region. On the contrary, genotype A was found only in two districts in Irkutsk province and three districts in Altai region. Despite the significant predominance of genotype E in most of the studied areas, only genotype A of *T. equi* was detected in the Ust-Koksa district in the Republic of Altai (Fig. 1; Supplementary Table S1).

All sequences belonging to genotype E were identical to the widespread *T. equi* variant, which was found in equines from China (KF559357, MH651213, MT093500, MT093500), South Korea (HM229408), Mongolia (LC781881, LC781885, LC781912), and Kazakhstan (MN857677). Of 32 samples of *T. equi* belonging to genotype A, 30 samples were identical to each other and to a specimen identified in equine blood from China (MT093496), whereas specimens Irk-Ecab_80 and Alt-Ecab_1/7 differed

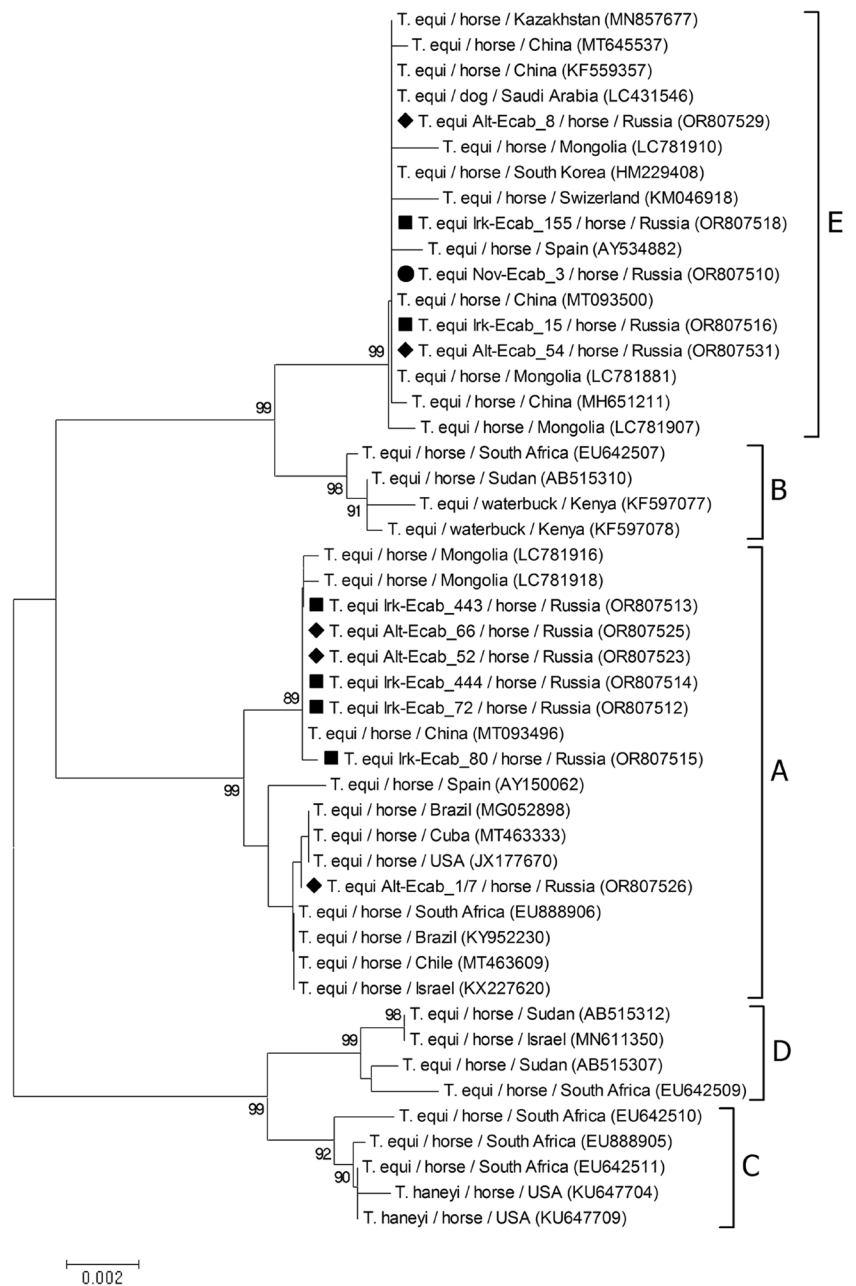
from them by one and seven nucleotide substitutions, demonstrating 99.9 and 99.4% identity, respectively.

The Irk-Ecab_80 sequence differed from all known sequences in the GenBank database. The Alt-Ecab_1/7 sequence has one polymorphic site, indicating the presence of mixed infection caused by two *T. equi* sequence variants. Notably, both sequence variants corresponded to the sequences previously found in equine blood from different regions: South Africa (EU888906), USA (JX177670), Brazil (KJ573370, KU240064, KY464029, KY952230, MG052898), Chile (MT463609), Cuba (MT463333, KY111762), Israel (KX227620, MK392050, MK063842, MN611318), and Iran (MK615933) (Fig. 2).

Babesia caballi genetic variability

Of 54 *B. caballi* specimens, 53 samples were genetically characterized by the 18S rRNA gene; for 28 specimens

Fig. 2 The phylogenetic tree constructed by the ML method based on sequences of the 1195 bp fragment of the 18S rRNA gene of *T. equi*. The specimens from this study from different regions are marked by ●—Novosibirsk province, ■—Irkutsk province, ◆—the Republic of Altai. Genotypes of *T. equi* (A–E) are given according to Bhoora et al. (2020). The scale bar indicates an evolutionary distance of 0.005 nucleotides per position in the sequence. Significant bootstrapping values (>80%) are shown on the nodes



from different locations, 1146–1162 bp fragments were sequenced. Seven sequence variants (v1–v7) of *B. caballi* were identified; these variants differed between themselves by 1–13 mismatches (98.9–99.9% identity). Of them, variant v1 corresponded to *B. caballi* sequences from different regions, namely, China (MH651219, MN156287), Brazil (KY952236), Cuba (MT463341), and South Africa (EU642512) and from ixodid ticks from China (MN163022) and Kazakhstan (MN907450). Variant v4 was identical to *B. caballi* sequences from equine blood in China (MZ331329, MZ331331, MZ331349, and MZ331354). Sequences of other five variants differed from all known *B. caballi* sequences from the GenBank database. The distribution

of *B. caballi* variants varied depending on geography. The greatest genetic diversity of *B. caballi* was observed in Altai region, where six variants (v1–v4, v6, and v7) were identified, while only three variants (v4, v5, and v7) were detected in Irkutsk province. In Novosibirsk province, the only *B. caballi* specimen corresponded to the widespread variant v1 (Table 3, Fig. 3). In some cases, the correlation has been observed between a particular *B. caballi* variant and location. Thus, the unique *B. caballi* variant v5 was found only in Bokhansky district of Irkutsk province, and in this district, variant v5 was found in four of five infected horses including cases of mixed infection (Supplementary Table S1).

Table 3 The results of genotyping of *T. equi* and *B. caballi*

Region	<i>T. equi</i>			<i>B. caballi</i>		
	No. of positive samples	No. of genotyped samples	<i>T. equi</i> genotypes (n)*	No. of positive samples	No. of genotyped samples	<i>B. caballi</i> variants (n)*
Novosibirsk province	11	9	E (n=9)	1	1	v1 (n=1)
Irkutsk province	281	145	E (n=122) A (n=23)	22	22	v4 (n=16) v5 (n=1) v4/5 [#] (n=3) v4/7 [#] (n=2)
Republic of Altai	161	127	E (n=118) A (n=9)	31	30	v1 (n=6) v2 (n=2) v3 (n=1) v4 (n=8) v6 (n=1) v1/2 [#] (n=3) v2/3 [#] (n=2) v4/7 [#] (n=7)
Total	453	281	E (n=249) A (n=32)	54	53	v1 (n=7) v2 (n=2) v3 (n=1) v4 (n=24) v5 (n=1) v6 (n=1) v1/2[#] (n=3) v2/3[#] (n=2) v4/5[#] (n=3) v4/7[#] (n=9)

(n)*—number of samples with the certain *T. equi* genotype or *B. caballi* variant

v4/7[#]—samples containing two *B. caballi* variants

Cases of mixed infection with two *B. caballi* variants were established based on the presence of polymorphic sites at positions characteristic of the identified *B. caballi* variants. As a result, four variants of mixed infections (v1/v2, v2/v3, v4/v5, and v4/v7) were detected. Cases of mixed infection with two *B. caballi* variants were detected in 5 of 22 infected horses in Irkutsk province and in 12 of 30 infected horses in Altai region (Table 3). Considering mixed infections, *B. caballi* variant v4 appeared to be the most common and widespread; it was detected in 21 of 22 horse blood samples from six districts of the Irkutsk region and in 15 of 30 blood samples from four districts of the Republic of Altai (Table 3, Supplementary Table S1).

Phylogenetic analysis demonstrated that the determined *B. caballi* sequences belong to four distinct branches within genotype A (Fig. 3). The first branch was formed by *B. caballi* variant v1 altogether with previously described *B. caballi* sequences from different regions. The second branch was formed by *B. caballi* variants v4–v5 and sequences from equine blood from China. Two remaining branches

contained only *B. caballi* sequences from this study, belonging to variants v2–v3 (a branch 3) and v6–v7 (a branch 4) (Fig. 3).

To establish phylogenetic positions of identified *B. caballi* sequences, we analyzed all available *B. caballi* sequences of the 18S rRNA gene with a length more than 900 bp from the GenBank database and *B. caballi* sequences from this study. The reconstructed phylogenetic tree contained five monophyletic clusters (designated A1–A5) within genotype A and three monophyletic clusters (B1–B3) within genotype B (Fig. 4). Cluster A1 was formed by 55 sequences from remote regions of Europe, Asia, Africa, and Americas, including *B. caballi* variant v1 from this study. Cluster A2 contained 53 sequences from Russian Siberia (variants v4 and v5) and China. Each of clusters A3 and A4 contained three sequences from this study (variants v2, v3 and v6, v7) and cluster A5 was formed by 11 sequences from Chinese pikas and a vole. A new cluster B3 contained two *B. caballi* sequences from Guinea ticks (Fig. 4).

Fig. 3 The phylogenetic tree constructed by the ML method based on sequences of the 933 bp fragment of the 18S rRNA gene of *B. caballi*. The specimens from this study from different regions are marked by ●—Novosibirsk province, ■—Irkutsk province, ◆—the Republic of Altai. Genotypes of *B. caballi* (A and B) are given according to Bhoora et al. (2020). *Babesia caballi* sequences determined in this study were subdivided into branches 1–4. The scale bar indicates an evolutionary distance of 0.005 nucleotides per position in the sequence. Significant bootstrapping values (> 80%) are shown on the nodes. *Babesia gibsoni* was used as an outgroup

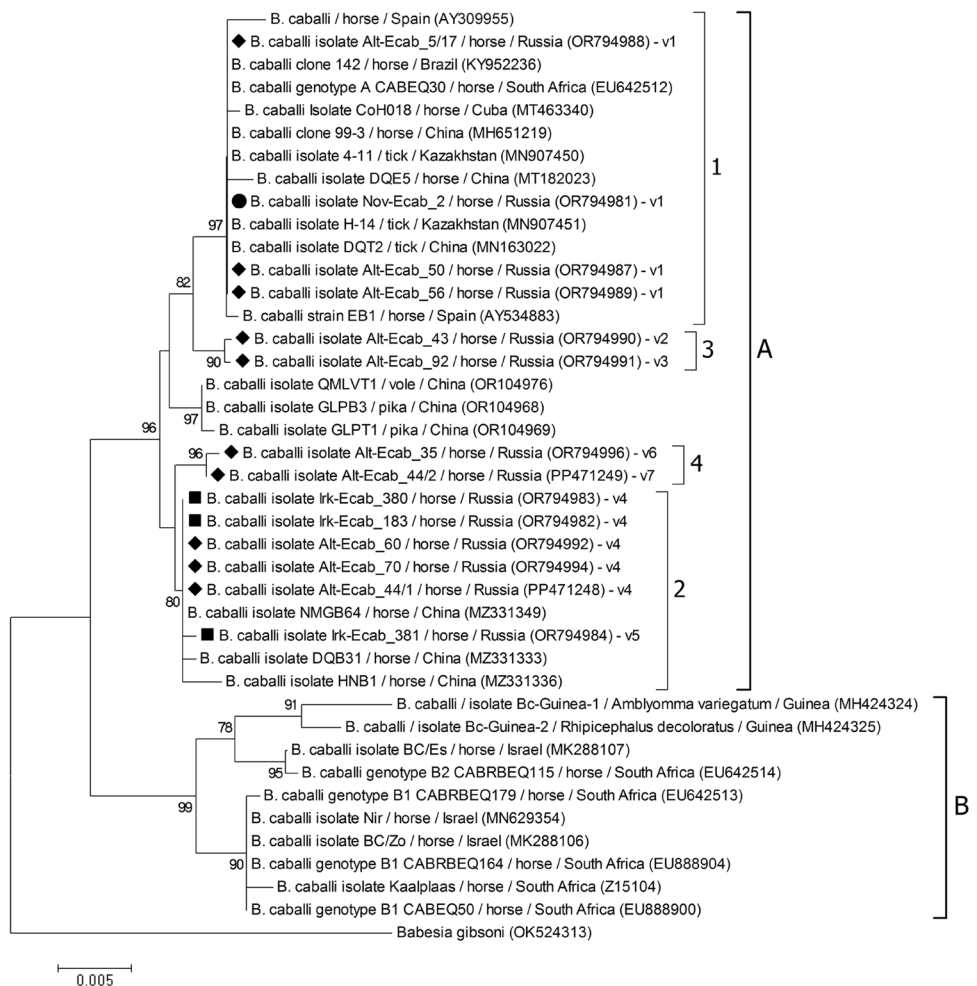
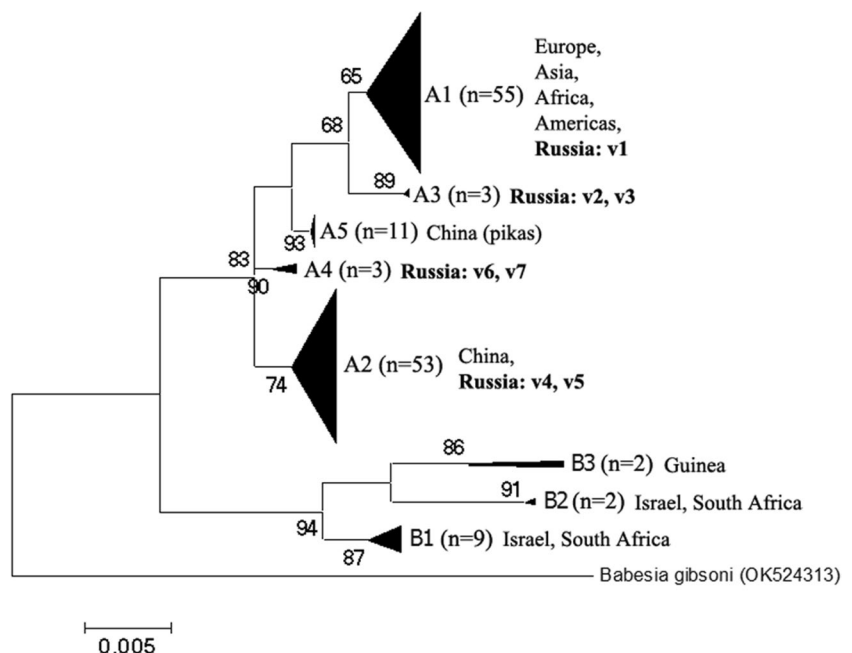


Fig. 4 The compressed phylogenetic tree constructed by the ML method based on 933 bp fragment of the 18S rRNA gene of *B. caballi*. Phylogenetic analysis included all sequences available up to March 2024. Phylogenetic clusters determined in this study were designated as A1–A5 and B1–B3. The numbers in parentheses indicate the number of samples belonging to the certain cluster. The scale bar indicates an evolutionary distance of 0.005 nucleotides per position in the sequence. Significant bootstrapping values (> 65%) are shown on the nodes. *Babesia gibsoni* was used as an outgroup



Discussion

In this study, the prevalence and genetic diversity of EP pathogens in Russia were investigated using molecular methods for the first time. The study involved 750 horses from three remote regions of Siberia: Novosibirsk province, Irkutsk province, and Altai region, which includes the Republic of Altai and the Altai Territory (Fig. 1). The Republic of Altai is the territory with the largest number of horses among the Siberian provinces; in 2014, more than 130,000 horses were recorded here. In contrast, only 66,900, 36,000, and 33,500 horses were registered in the Altai Territory, Irkutsk province, and Novosibirsk province, respectively (Kniazev and Timchenko 2016). In all regions, horses are bred for sport, tourism, and recreation. In addition, local national minorities (Altaians and Buryats) breed horses for meat production in the Republic of Altai and Irkutsk province; in these regions, horses often graze freely. However, regardless of the purposes of breeding horses and the conditions of their keeping, all horses can have frequent contacts with ticks during grazing and, despite antiprotozoal treatment, can receive EP pathogens.

Sampling was carried out in different parts of the Republic of Altai and in one district of the Altai Territory adjacent to the Republic of Altai. In Irkutsk province, the samples were collected in its southeastern part, where the number of horses was the greatest (Fig. 1). In both regions, horses from private farms, equestrian centers, and racetracks were studied. In Novosibirsk province, all examined horses were from private stables. Despite the large distance between the sampling areas and the difference in their natural and climatic conditions, the prevalence of EP pathogens in horses was similar in different regions and exceeded 50%. However, some sampling areas were free of EP (Supplementary Table S1). In at least some cases, the absence of infected horses was related to the absence of specific vectors. Thus, at sampling points in the Charyshsky district of the Altai Territory, the population of ixodid ticks was represented predominantly by *Ixodes* spp., but not by *Dermacentor* spp., which are the most likely carriers of EP pathogens in Siberia (Budnik 1955; Ovchinnikov et al. 1941).

The obtained results demonstrated that significant parts of the Republic of Altai, Irkutsk province, and, possibly, Novosibirsk province are endemic for EP and therefore preventive measures are necessary. Particular attention should also be paid to locations where agents of EP have not been found, since a possible change in the species composition of ticks in EP-free areas can lead to an outbreak of EP. In all studied regions, two causative agents of EP were identified—*T. equi* and *B. caballi*, and the prevalence of *T. equi* was significantly higher compared to *B. caballi* (Table 2). Similar high prevalences of EP were observed in most endemic areas of South Africa, Cuba, Brazil, Mongolia, and China, and in

most areas, horses were also more likely to be infected with *T. equi* than with *B. caballi* (Bhoora et al. 2020; Chen et al. 2022; Díaz-Sánchez et al. 2018; Otgonsuren et al. 2024; Qablan et al. 2013; Tirosh-Levy et al. 2020a). This may be due to the fact that *T. equi* can persist in the blood of horses throughout their lives, while *B. caballi* can only persist for several years (Scoles and Ueti 2015).

This study provides the first data concerning genetic diversity of both *T. equi* and *B. caballi* in Russia. Despite *T. equi* being shown to be a genetically variable species (Bhoora et al. 2009; Díaz-Sánchez et al. 2018; Tirosh-Levy et al. 2020a, b; Seo et al. 2013; Wang et al. 2019), the analyzed *T. equi* specimens from Siberia were highly conserved and belonged only to genotypes A and E among five previously described *T. equi* genotypes. *Theileria equi* genotype A is the most widespread in the world; however, in all studied regions of Siberia genotype E was predominant. Similarly, genotype E was predominant in equine blood in various regions of nearby China and Mongolia, where it was identified in 93–95% of infected horses (Chen et al. 2022; Otgonsuren et al. 2024).

With two exceptions, the determined sequences of *T. equi* were identical within each genotype. Notably, the single identified sequence variant of genotype E was identical to widespread *T. equi* variant from neighboring China, Kazakhstan, Mongolia, and South Korea (Otgonsuren et al. 2024; Seo et al. 2013; Wang et al. 2019), whereas the predominant in Siberia sequence variant of genotype A was quite rare worldwide and corresponded to a single *T. equi* sequence from China (MT093496) (Fig. 2). The observed high genetic stability of *T. equi* isolates from Siberia suggests that *T. equi* infection in the studied regions was spread from a limited number of infected horses. Notably, an isolate Alt-Ecab_1/7, which significantly differed from other Siberian specimens, corresponded to a large number of *T. equi* sequences from geographically distant regions: South Africa, Americas, Israel, and Iran, but not from neighboring regions of China, Mongolia, or Kazakhstan (Bhoora et al. 2009; Díaz-Sánchez et al. 2018; Hall et al. 2013; Margalit Levi et al. 2018; Tirosh-Levy et al. 2020b). This discrepancy may be due to the fact that Alt-Ecab_1/7 sequence was found in a horse from an equestrian school (Gorno-Altai), which could have been imported from a region distant from Altai.

In contrast to *T. equi*, the examined isolates of *B. caballi* were significantly more variable. Seven sequence variants of *B. caballi* belonging to genotype A were discovered; five of them differed from previously known sequences (Bhoora et al. 2009; Chen et al. 2022; Díaz-Sánchez et al. 2018; Wang et al. 2019) and formed two well-supported branches on the phylogenetic tree (Fig. 3).

It is generally accepted that *B. caballi* sequences are subdivided into genotypes A, B1, and B2 (Bhoora et al. 2009,

2020; Tirosh-Levy et al. 2020a). Previously, the genetic heterogeneity of genotype A was demonstrated in a study of Nehra et al. (2021), in which, based on the analysis of 300 bp fragments, two *B. caballi* specimens from a horse and a tick from China were assigned to a separate clade. Later, a large number of new *B. caballi* sequences were submitted to the GenBank database (Chen et al. 2022; Kartashov et al. 2021). Therefore, to explore the phylogeny of *B. caballi* given the latest sequences, we performed a phylogenetic analysis based on all *B. caballi* 18S rRNA gene sequences (> 900 bp) available up to March 2024, including sequences determined in this study. The heterogeneity of genotype A turned out to be significantly higher than previously considered. It was first demonstrated that genotype A can be subdivided into five well-supported clusters; we propose to designate them as clusters A1–A5. Clusters A1 and A2 included the majority of known sequences (more than 50 sequences in each cluster), whereas each of other clusters includes 3–11 sequences. Notably, cluster A1 included sequences from different continents (Bhoora et al. 2009; Díaz-Sánchez et al. 2018; Wang et al. 2019), while cluster A2 contained only sequences from China (Chen et al. 2022) and Russian Siberia (this study). The minor clusters A3 and A4 were formed by sequences from Siberia, and cluster A5 contained *B. caballi* sequences from atypical hosts—Chinese pikas and voles (Fig. 4). Notably, based on analysis of shorter sequences, cluster A5 probably also includes Chinese isolates from a horse and a tick (Nehra et al. 2021). As for genotype B, in addition to the previously described clusters B1 and B2, it contains the third cluster, B3, formed by *B. caballi* sequences from *Amblyomma variegatum* and *Rhipicephalus decoloratus* from Guinea (Kartashov et al. 2021).

Conclusion

In this study, the genetic diversity of EP agents in Russian Siberia was first demonstrated. The identified *T. equi* sequences were highly conserved and belonged to genotypes A and E. The determined *B. caballi* sequences were more variable and belonged to four branches within genotype A. Seven sequence variants of *B. caballi* were found, and five of them were identified for the first time. The performed phylogenetic analysis of all available *B. caballi* sequences first demonstrated that genotype A of *B. caballi* can be reliably subdivided into five clusters, A1–A5. Thus, the conducted genetic study of *B. caballi* from Siberia significantly expanded the data on the genetic diversity of this agent.

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Author contribution VR: conceptualization, methodology, investigation, formal analysis, writing original draft. VM: sample collection, data analysis, funding acquisition. OS: sample collection, investigation,

visualization. TE: investigation, data analysis. AT: investigation, formal analysis. IM: sample collection. VF: investigation. YI: investigation. IK: methodology, resources, funding acquisition. NT: conceptualization, funding acquisition, review and editing manuscript. All authors read and approved the final version of the manuscript.

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Data availability Sequence data that support the findings of this study were submitted to the GenBank database under accession numbers MG551915, OM475522-OM475525, and OR807510-OR807531 for *T. equi* and OR794981-OR794998 and PP471248-PP471249 for *B. caballi*.

Declarations

Ethics approval All experiments with animals were conducted according to the guidelines for experiments with laboratory animals (Supplement to the Order of the Russian Ministry of Health, no. 755, of August 12, 1977). Animal use and experimental procedures were approved by the Bioethical Committee of the Scientific Center of Family Health Problems and Human Reproduction, Irkutsk, Russia (Protocol No. 5, 26.10.2012 and Protocol No. 2, 18.02.2020).

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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