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Genotyping of *Borrelia*, *Rickettsia* and *Anaplasma* in *Ixodes ricinus* and *Dermacentor reticulatus* ticks in the Kaliningrad region

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Abstract

Background. Tick-borne bacterial and protozoal pathogens pose a significant public health problem. The aim of this study was to detect and genotype Borrelia, Rickettsia and Anaplasma in *Ixodes ricinus* and *Dermacentor reticulatus* ticks collected in the Kaliningrad region in 2021–2022.

Materials and methods. The study included 862 *I. ricinus* and 803 *D. reticulatus* ticks (1665 in total) collected in 33 biotopes of the Kaliningrad region. Detection of the DNA of tick-borne pathogens was carried out in individual ticks by PCR using a set of specific primers, followed by sequencing and phylogenetic analysis.

Results. The level of infection of *I. ricinus* ticks with Borrelia was 15.5%, and genotyping by the *p66* gene sequence showed the presence of genetic material from four species: *B. afzelii, B. garinii, B. valaisiana,* and *B. lusitaniae*. In *D. reticulatus* ticks, no Borrelia genetic material was detected. The Rickettsia DNA has been found in both tick species. Moreover, the infection rate of *I. ricinus* ticks was 2.6%, and *D. reticulatus* — 21.2%. *R. helvetica* were found in *I. ricinus* ticks, and *R. raoultii* in meadow ticks when genotyping by *gltA* gene. Genetic markers of *Anaplasma phagocytophilum* have been found in *I. ricinus* and *D. reticulatus* ticks. Cases of co-infection of an individual tick have also been identified.

Conclusion. Six different species of tick-borne pathogens were found in the *I. ricinus* and *D. reticulatus* ticks collected in the Kaliningrad region and *R. helvetica*, *R. raoultii* and *A. phagocytophilum* were identified for the first time.

Keyworlds: *ixodes ticks, tick-borne infections, Borrelia, Rickettsiae, Anaplasma, genotyping, phylogenetic analysis, Kaliningrad region, Russia*

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Оригинальное исследование
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Генотипирование боррелий, риккетсий и анаплазм в клещах *Ixodes ricinus* и *Dermacentor reticulatus* в Калининградской области

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Аннотация

Актуальность. Возбудители клещевых инфекций бактериальной и протозойной природы представляют существенную проблему для общественного здравоохранения.

Цель исследования состояла в детекции и генотипировании боррелий, риккетсий и анаплазм в клещах *Ixodes ricinus* и *Dermacentor reticulatus*, собранных на территории Калининградской области в 2021–2022 гг.

Материалы и методы. В исследование были включены 1665 клещей: *I. ricinus* (*n* = 862) и *D. reticulatus* (*n* = 803), собранных в 33 биотопах Калининградской области. Детекцию генетического материала клещевых патогенов проводили в индивидуальных клещах методом ПЦР с последующим секвенированием и филогенетическим анализом специфических последовательностей ДНК.

Результаты. Уровень инфицированности клещей *I. ricinus* боррелиями составил 15,5%, причём генотипирование по последовательности гена *p66* показало наличие ДНК боррелий четырех видов: *Borrelia afzelii, B. garinii, B. valaisiana* и *B. lusitaniae*. В клещах *D. reticulatus* ДНК боррелий не выявлено. Генетический материал *Rickettsia* spp. был обнаружен в обоих видах клещей, причём уровень инфицированности клещей *I. ricinus* составил 2,6%, а *D. reticulatus* — 21,2%. В клещах *I. ricinus* обнаружены риккетсии *R. helvetica,* а в луговых клещах — *R. raoultii* при проведении их генотипирования по гену *gltA.* ДНК *Anaplasma phagocytophilum* были обнаружены как в клещах *I. ricinus,* так и в клещах *D. reticulatus.* Выявлены также случаи коинфицирования индивидуального клеща несколькими клещевыми патогенами.

Заключение. В клещах *I. ricinus* и *D. reticulatus,* собранных на территории Калининградской области, обнаружены 6 видов возбудителей клещевых инфекций бактериальной и протозойной природы, причём *R. helvetica, R. raoultii* и *A. phagocytophilum* были выявлены впервые.

Ключевые слова: *иксодовые клещи, клещевые инфекции, боррелии, риккетсии, анаплазмы, генотипирование, филогенетический анализ, Калининградская область, Россия*

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Ticks can be infected with pathogens of viral, bacterial and protozoan nature [1–3]. In addition to fairly well-studied pathogens, which include tick-borne encephalitis virus and ixoid borreliosis pathogens, other microorganisms that cause human diseases may be present in ticks, including in European countries [2, 4–6]. Tick-borne infections are a common group of zooanthroponotic diseases in Russia [7, 8]. The structure and characterization of tick-borne infections, including genotyping of their pathogens, in the European part of Russia is not sufficiently studied [9]. In recent years, cases of human tick-borne infections have been associated with ticks *Ixodes persulcatus* (Schulze, 1930), *I. pavlovskyi* (Pomerantzev, 1946), *Dermacentor reticulatus* (Fabric, 1794), *D. marginatus* (Sulzer, 1776), and *D. nuttali* (Olenev, 1928) in Siberian and Far Eastern regions of Russia [8, 10]. In the European parts of Russia, *I. ricinus* ticks are widely distributed (Linnaeus, 1758), which are prevalent in the western regions of the country. Interestingly, the appearance of *D. reticulatus* ticks is noted in urban and suburban biotopes [8, 11].

Primers using for isolation gene fragments of *Borrelia*, *Rickettsia* and *Anaplasma* from ixodes ticks

Primer	Primer sequence (5′→3′)	Temperature, ºC	Size, bp	Reference
Borr2rF	CGAATTAGGCAAAGACGATCC	56	548	[8]
Borr2rR	TTTCCATAAGCTCCTGATAAGCCA			
CS409d	CCTATGGCTATTATGCTTGC	56	769	[16]
RP1258n	ATTGCAAAAAGTACAGTGAACA			
MSP2-3f	CCAGCGTTTAGCAAGATAAGAG	55	334	[17]
MSP2-3r	GCCCAGTAACAACATCATAAGC			

For example, in Tomsk, their numbers increased more than 200 times for urban biotopes, and the infection rate of *D. reticulatus* ticks was approximately 44–48% for *Rickettsia* spp., 0.7–0.9% for tick-borne encephalitis virus, and 0.6% for *Anaplasma phagocytophilum*.

Earlier in *I. ricinus* ticks located in the Leningrad and Kaliningrad regions, 4 species of Borrelia were presumably detected by PCR at an infection rate of 11.5% [12]. Later, taiga ticks (*I. persulcatus*) were detected in park areas of St. Petersburg on the Baltic Sea coast with an infection rate of 9.3% with borreliae genotyped as *B. afzelii* and *B. garinii* [13]. In Finland, the infection rate of *I. ricinus* and *I. persulcatus* infected with various tick-borne pathogens reached 30% with a significant predominance of *Borrelia burgdorferi sensu* lato [14]. At the same time, *I. ricinus* ticks were significantly more frequently infected and co-infected with various bacterial and protozoan pathogens.

In the Kaliningrad region in 2022, 5379 people sought medical help due to tick bites[1]. Only cases of viral tick-borne encephalitis and ixoid tick-borreliosis are diagnosed annually in patients who are traditionally associated with *I. ricinus* ticks: 3 cases of tick-borne encephalitis and 49 cases of tick-borreliosis were reported in 2022. The circulation of other tick-borne pathogens and their species affiliation have not been described.

The aim of this study was to detect, study species affiliation and genotyping of Borrelia, Rickettsia and Anaplasma detected in ixodid ticks collected in different biotopes of the Kaliningrad region.

Materials and methods

Mites were collected from vegetation using the "per flag" method in different biotopes of the Kaliningrad region in 2021–2022. Geographical coordinates of biotopes and the number of mites collected in biotopes are presented in the Appendix on the journal's website. Species identification of ticks was carried out by morphological method [15].

[1] State report "On the state of sanitary and epidemiological changes in the population in the Kaliningrad region in 2022." Kaliningrad; 2023. 238 p. URL: https://39.rospotrebnadzor.ru/sites/default/files/doklad_o_goskontrole_za_2022_kaliningradskaya.pdf

Nucleic acid isolation

Ticks were treated twice with 70% ethanol to inactivate infectious agents and washed with phosphate-buffered saline. Homogenization of the obtained samples was performed using a laboratory homogenizer TissueLyserLT (Qiagen) in 300 µl of sterile physiological saline. The nucleic acid isolation was performed from 100 µl of homogenate using the AmpliPrime RIBO-prep reagent kit (NextBio) according to the manufacturer's instructions.

PCR testing

Screening of the obtained samples for the presence of genetic markers of the studied pathogens was carried out by PCR using specific primers (Table) on a thermocycler T-1000 (Bio-Rad) in 25 µl of the reaction mixture of the following composition: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 mM of each primer, 1.5 units of HS-Taq polymerase activity (Eurogen) and 1-100 ng of DNA matrix. The following temperature regimes were used for PCR: preliminary activation of polymerase — 95ºC for 5 min; 38 cycles: 95ºC — 20 s, T_{anneal} — 20 s, 72ºC — 1 min; final elongation at 72ºC — 4 min.

The amplicons were detected by gel electrophoresis in 2% agarose gel in Tris-acetate buffer containing 0.1% ethidium bromide. Amplification products were purified from agarose gel using a microcolumn-based kit (Biosilica).

Sequencing and nucleotide sequence analysis

Sanger sequencing was performed using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). Nucleotide sequences were determined by capillary electrophoresis using a 3130xl Genetic Analyzer automatic sequencer (Applied Biosystems). Analysis of the obtained nucleotide sequences was performed using the UniproUGENE v. 1.46 program. The obtained nucleotide sequences were compared with previously published sequences in GenBank using the BLAST search application. The nucleotide sequences were aligned using the MUSCLE algorithm in the MEGA X software. Phylogenetic analysis of nucleotide sequences was performed by the maximum likelihood

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method using the Tamura-Nei evolution model to analyze genetic relationships/clustering of nucleotide sequences. The statistical reliability indices of phylogenetic tree nodes were calculated by bootstrap analysis using 1000 random replicates.

Deposit of nucleotide sequences

The following nucleotide sequences were deposited in the GenBank database: fragments of the *msp2* gene of *A. phagocytophilum* (OR488786-OR488799); *p66* gene fragments of Borrelia: *B. afzelii* (OR488840-OR488890), *B. garinii* (OR488891-OR488929), *B. valaisiana* (OR488930-OR488948), *B. lusitaniae* (OR48898949-OR488967); Rickettsiae *gltA* gene fragments: *R. helvetica* (OR496610-OR496611) and *R. conorii* subsp. *raoultii*, hereafter as the basionym of *R. raoultii* (OR496612-OR496613).

The studies were conducted in compliance with the biosafety rules regulated in MU 1.3.2569-09, SP 1.3.3118-13, SP 3.1.3310-15.

Results

We collected and analyzed 1665 individual samples of nymphs and imago of *I. ricinus* (n = 862) and *D. reticulatus* (*n* = 803) ticks from 33 urban, suburban and characteristic natural biotopes of the Kaliningrad region (**Fig. 1**). The biotopes studied were subdivided according to the species of ticks collected in them as follows: 11 biotopes in which only *I. ricinus* ticks, 9 biotopes with *D. reticulatus* ticks and 13 biotopes with 2 tick species. Quite unusually, in fact, half of all collected mites were attributed to the meadow mite, which was found in 2/3 of the biotopes studied and absolutely prevailed in 9 of them.

The infection rate of *I. ricinus* Borrelia infection was 15.5% (128/862; 95% CI 13.2–18.1). Determination of the nucleotide sequence of a fragment of the *p66* gene with a length of about 560 bp among 128 samples revealed Borrelia of four species from the *B. burgdorferi s.l.* complex: in 51 ticks, DNA of *B. afzelii* (39.9%; 95% CI 31.8–48.5), in 39 — *B. garinii* (30.5%; 95% CI 23.2–38.5), in 19 — *B. valaisiana* (14.8%; 95% CI 9.7–22.0), in 19 — *B. lusitaniae* (14.8%; 95% CI 9.7–22.0) were detected. No Borrelia genetic material was detected among the studied samples of ticks of *D. reticulatus* species. Phylogenetic analysis showed that Borreliae detected in *I. ricinus* ticks in the territory of the Kaliningrad region cluster with prototypic sequences isolated primarily in European countries (**Fig. 2**). Sequence analysis of the fragment of the *p66* gene of *B. afzelii* revealed 6 alleles of this gene, for *B. garinii* isolates 8 allelic variants differing from each other by 1-14 nucleotide substitutions were detected, and *B. valaisiana* and *B. lusitaniae* isolates had 2 and 4 substitutions.

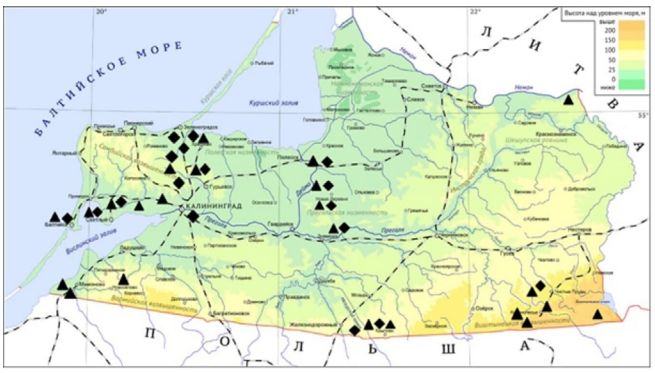

Fig. 1. Locations of biotopes in the Kaliningrad region, where ixodid ticks were collected in 2021–2022.

The rickettsial infection rate in ticks was 11.5% (191/1665; 95% CI 10.2–13.1%). Among *I. ricinus* ticks, Rickettsia DNA was detected in 22 samples and the infection rate was 2.6% (22/862; 95% CI 1.7–3.8%). All identified Rickettsia isolates from *I. ricinus* ticks were attributed to *R. helvetica* by a fragment of the citrate synthase gene (*gltA*). When analyzing the nucleotide sequences of the *gltA* gene, two main genetic variants of *R. helvetica* circulating in the Kaliningrad region were identified. They differ from each other by 2 synonymous nucleotide substitutions (the level of homology between the genetic variants is 99.8%). One of the genetic variants corresponds to the previously described variants of *R. helvetica* found in the Komi Republic and Omsk region [9, 18], the other genetic variant differs from the known sequences.

In 21.1% of *D. reticulatus* ticks, Rickettsia DNA was detected (169/803; 95% CI 18.4–24.0%), which was genotyped as *R. raoultii*. Phylogenetic analysis of *R. raoultii* revealed the existence of two variants differing by a single synonymous substitution (**Fig. 3**). In general, these genetic variants correspond to a wide range of *R. raoultii* isolates circulating in Europe, Russia and China [19, 20].

A. phagocytophilum DNA was detected by PCR in 12 samples of ticks of the species I. ricinus (1.4%; 95% CI 0.8–2.5%) and in 2 samples of *D. reticulatus* ticks (0.2%; 95% CI 0.1–0.9%). The nucleotide sequences of the *msp2* gene fragment of approximately 340 bp in length were determined in the detected *A. phagocytophilum* isolates, followed by phylogenetic analysis (**Fig. 4**). Three *A. phagocytophilum* genetic variants with a homology level of 98.6%, identical or closest to *A. phagocytophilum* isolates circulating in Norway and Poland, were detected in the Kaliningrad region.

Two tick samples contained simultaneously genetic material of *B. afzelii* and *R. helvetica*, one tick sample contained DNA of *B. valaisiana* and *A. phagocytophilum.*

Discussion

The results of regular long-term field observations show that the main recreational landscapes of the Kaliningrad region, including landscapes of the Baltic Sea coast, have established populations of ixodid ticks. The

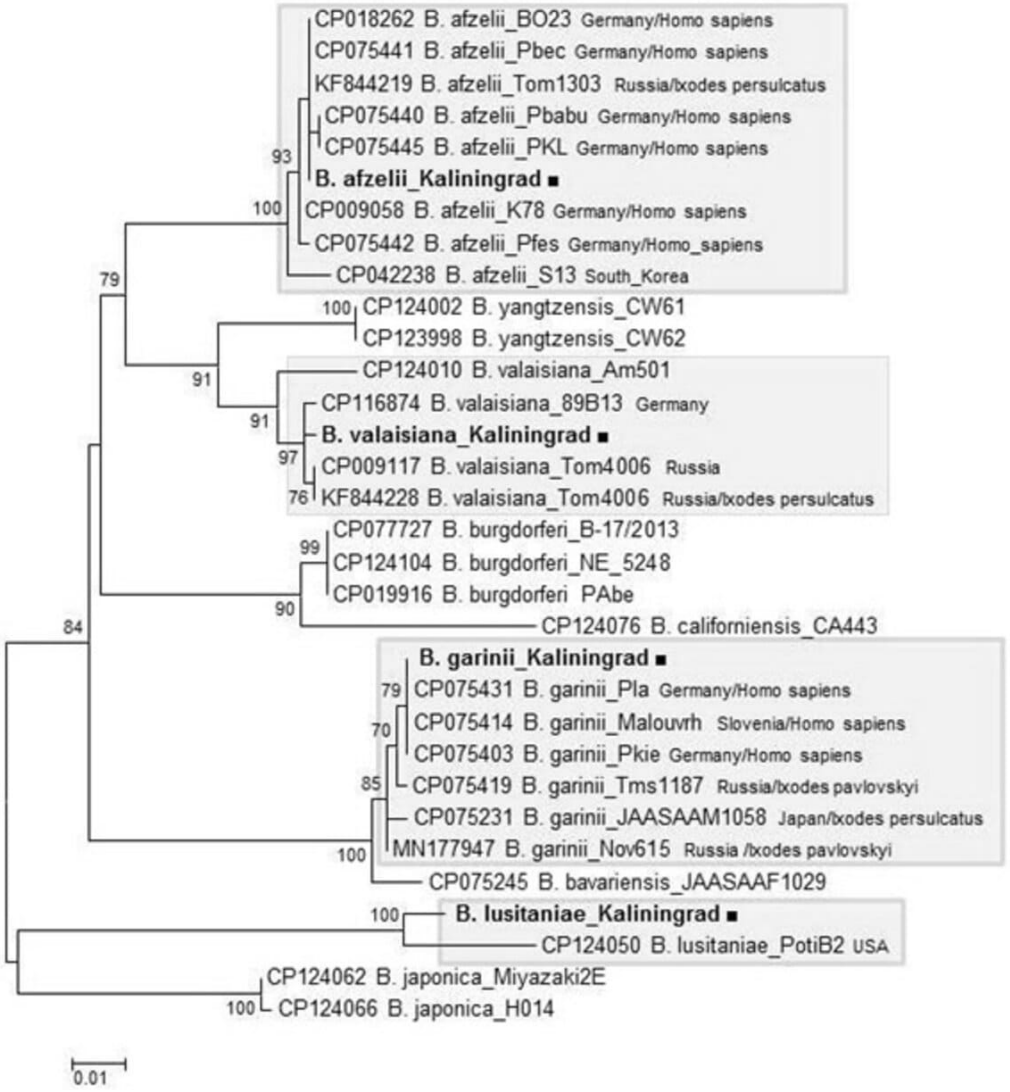

Fig. 2. Dendrogram of nucleotide sequences of the *p66* gene fragment for detected borrelia isolates.
Black squares — sequences derived from *I. ricinus* tick.

activity of ixodids in zones with pronounced anthropogenic load is significantly higher than in similar landscapes with insignificant anthropogenic load. Thus, in recent years, 4194–7300 people seek medical help for tick bites. Every year 2-16 cases of viral tick-borne encephalitis and 35-132 cases of ixoid tick-borne borreliosis are diagnosed. This repeatedly increases the risks of human contact with ixodid ticks, which can lead to human infection with various tick-borne pathogens.

Tick-borne borrelioses occupy an important place in the structure of infectious pathology in the Kaliningrad region. In ticks of the Kaliningrad region we detected and genotyped *B. afzelii*, *B. garinii* and *B. lusitaniae*, which are considered pathogenic for humans, and B. valaisiana, the pathogenicity of which is under discussion [21]. The infection rate (15.5%) correlates with earlier studies in the Leningrad and Kaliningrad regions [12]. *B. afzelii* and *B. garinii* are the most common pathogens of tick-borne borreliosis in humans and are most often found in *I. ricinus* ticks. Circulation of *B. lusitaniae* was shown for the first time in the territory of the Kaliningrad region. It should be noted that *B. lusitaniae* is mainly distributed in the countries of the Mediterranean region, such as Portugal, Morocco and Tunisia. In more northern latitudes, this pathogen was detected in Austria, Slovakia, Ukraine and Latvia [22].

No genetic markers of borreliosis could be detected in *D. reticulatus* ticks, although a very representative sample of ticks of this species was examined. Previously, a similar situation was recorded in Tomsk, where 315 ticks of this species collected in urban biotopes were individually examined [11]. In Tomsk urban biotopes, a more than 200-fold increase in the number of *D. reticulatus* ticks was actually detected during 2015. It was the explosive increase in the abundance of *D. reticulatus* which allowed to collect a significant number of these ticks in 2016–2017 and assess their role in the transmission of tick-borne infections in Tomsk.

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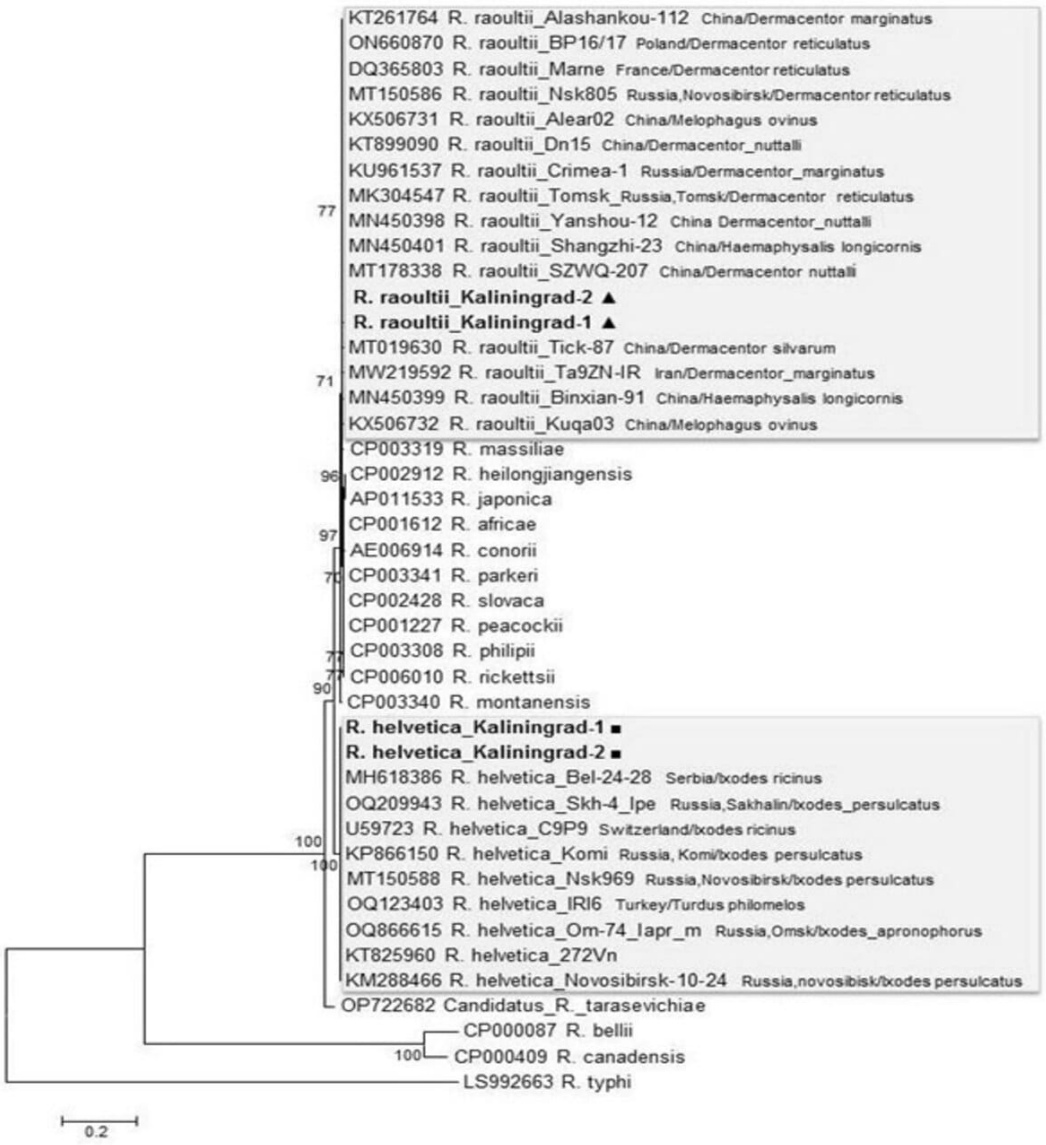

Fig. 3. Dendrogram of nucleotide sequences of the *gltA* gene fragment for identified *Rickettsia* isolates.
Black triangles — sequences derived from *D. reticulatus* tick; black squares — sequences derived from *I. ricinus* tick.

Rickettsiae carried by ixodid ticks are infectious agents capable of causing human disease. In the Kaliningrad region, we managed for the first time to establish the fact of circulation of two species of Rickettsia from the tick-borne spotted fever group: *R. helvetica* and *R. raoultii*. The registered level of infection of *I. r. raoultii* ticks with *R. helvetica* in *I. ricinus* ticks amounted to 2.6%. In other countries of the Baltic region it ranges from 5 to 10% [23]. However, ticks of *D. reticulatus* species were infected with *R. raoultii* in 21.2% of cases. At the same time, in Lithuania and Latvia the similar indicator reaches 38%, in Germany — 80% [24, 25]. In Russia, the level of infection of *D. reticulatus* with this Rickettsia species can vary in different regions from 21.9% to 45%. *R. helvetica* DNA was found in *I. persulcatus*, *I. ricinus*, *I. pavlovskyi* and *I. trianguliceps* ticks in different regions of northern Eurasia [10, 26, 27]. Human cases have been described, with patients with rickettsiosis caused by *R. helvetica* showing fever, rare rashes, and cases of perimyocarditis and meningitis described.

R. conorii subsp. *raoultii* was described as a new Rickettsia species in 2008 after the study of the prototype strain of *R. raoultii* isolated in 2005 in Kharbarovsk from *D. silvarum* ticks in Khabarovsk Krai [28]. In subsequent studies, *R. raoultii* was detected in *D. reticulatus*, *D. marginatus* and *D. nuttalli* ticks in a number of regions of the Asian part of Russia (Omsk region, Republic of Buryatia), Kazakhstan, China, and Mongolia [19, 20]. Rickettsiae genetically similar to *R. raoultii* have been identified in *Haemaphysalis hystricis* ticks in Japan and in *H. ornithophila*, *H. shimoga*, and *H. lagrangei* ticks in Thailand, as well as in *D. marginatus* ticks in Georgia, Turkey and European countries [29].

Serologic methods and DNA detection in the blood of patients confirmed the role of *R. raoultii* along with

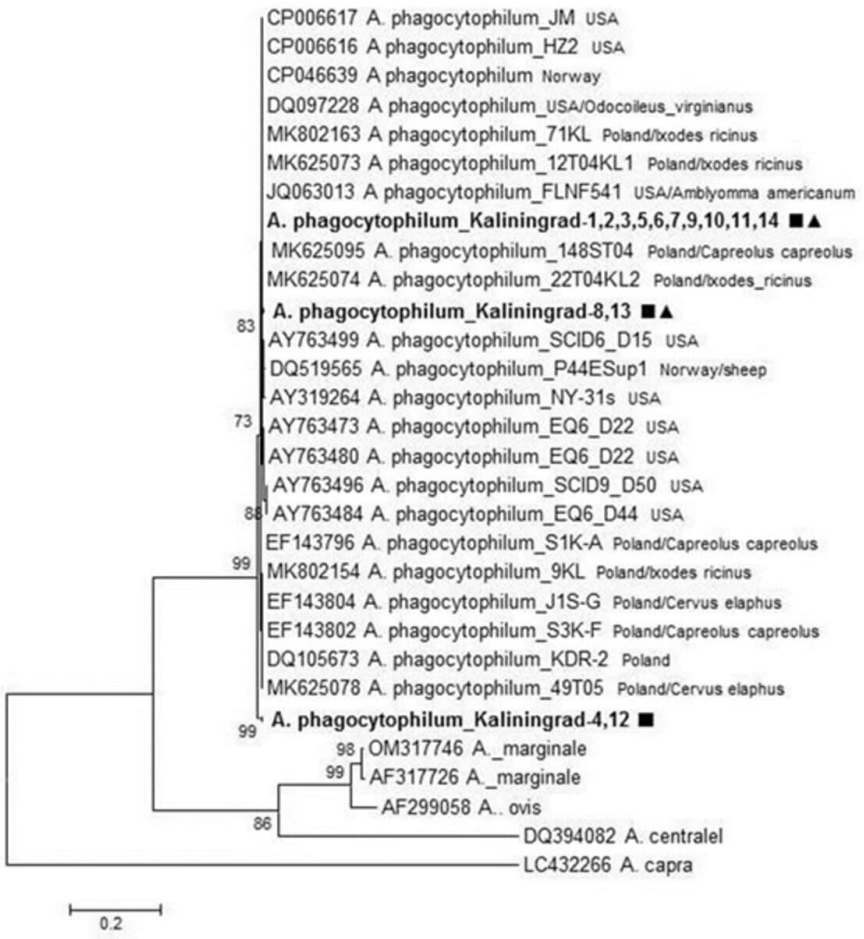

Fig. 4. Dendrogram of nucleotide sequences of the msp2 gene fragment for identified *A. phagocytophilum* isolates.
Black triangles — sequences derived from *D. reticulatus* ticks; black squares — sequence derived from *I. ricinus* ticks

R. slovaca as etiologic agents of TIBOLA syndrome, which is associated with *Dermacentor* spp. genus ticks [30]. Patients develop an asthenic syndrome, and fever (> 38ºC) is observed in a quarter of cases. For most patients, erythema persists for up to 1-2 months. If the tick bite is localized in the scalp, about one third of patients develop persistent baldness at the site of bite healing. At the same time, no cases of human rickettsioses in Kaliningrad region have been described so far.

The presence of *A. phagocytophilum* genetic material in *I. ricinus* and *D. reticulatus* ticks, revealed for the first time in this work, shows the active circulation of this pathogen in the Kaliningrad region. The level of infection of *D. reticulatus* and *I. ricinus* ticks (0.2% and 1.4%, respectively) corresponds to similar indicators for such countries as Denmark, Sweden, Norway and Germany, where the pathogen is found in 1–5% of ixodid ticks [23]. Phylogenetic analysis shows simultaneous circulation of at least 3 genetic variants of *A. phagocytophilum*. Human granulocytic anaplasmosis (HGA) was first described in the Russian Far East in 2000. Later confirmed cases of the disease were reported in Perm and Novosibirsk regions and in Altai. The clinical course of HGA is very polymorphic: from mild, subclinical forms to extremely severe, fatal cases, which account for 0.5–1.0% and are usually associated with the development of secondary infections. The disease is characterized by the appearance of headaches and muscle aches, the development of fever. Less than half of patients may have nausea, vomiting, anorexia, diarrhea, abdominal and joint pain, cough. In most cases, leukopenia, thrombocytopenia, and elevated serum levels of liver aminotransferases and C-reactive protein are noted in HGA patients. HGA disease has not been registered in the Kaliningrad region.

High level of infection of ixodal ticks with Rickettsia and Anaplasma in the Kaliningrad region, presence of constant contacts of population with ticks allows to expect occurrence of cases of infection of people with Rickettsia spp. of tick-borne spotted fever group and Anaplasma spp. Diagnosis of these diseases may be difficult due to the imperfection of their laboratory diagnostics. These infectious agents are not cultured by classical microbiological methods, and the genetic material of the pathogens can be detected in clinical material from patients over a very narrow time range. This

actualizes studies on seroepidemiological monitoring of these infections in the population living in the Kaliningrad region to clarify their current distribution.

Conclusion

In the collection of *I. ricinus* and *D. reticulatus* ticks, collected in 33 different biotopes in the Kaliningrad region in 2021–2022, DNA of 6 different species of tick-borne pathogens of bacterial and protozoan nature was detected. Sequencing of genome fragments of these pathogens and their phylogenetic analysis allowed to identify and genotype the following species of tick-borne pathogens: *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae*, *R. helvetica*, *R. raoultii* and *A. phagocytophilum*. *R. helvetica*, *R. raoultii* and *A. phagocytophilum* were detected for the first time in this region in both *I. ricinus* and *D. reticulatus* ticks. The obtained data confirm the necessity of continuous monitoring for circulation of pathogens of borreliosis, rickettsioses and anaplasmosis in natural foci of tick-borne infections in Kaliningrad region, further improvement of methods of diagnostics and prevention of these infections, including detection of possible cases of human cases of rickettsioses and HGA.

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