Вавиловский журнал генетики и селекции. 2020;24(1):55-59
DOI 10.18699/VJ20.595

Detection of *Ehrlichia* spp. and *Theileria* spp. in *Hyalomma anatolicum* ticks collected in Tajikistan

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Abstract. The objectives of our study were to survey the prevalence of genetic markers for *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. in *Hyalomma anatolicum* ticks collected in southwestern Tajikistan and to perform sequencing and phylogenetic analysis of fragments of the 16S rRNA gene and *groESL* operon from *Ehrlichia* spp. and fragments of the 18S rRNA gene of *Theileria* spp. detected in *H. anatolicum* ticks. *Hyalomma anatolicum* ticks collected in the Tursunzade and Rudaki districts of Tajikistan were tested for DNA of *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. by PCR with specific primers. The amplified fragments were sequenced and analyzed. DNA of *Ehrlichia* spp. (3.3 %) and *Theileria* spp. (3.3 %) was detected only in *H. anatolicum* ticks collected from the Rudaki district, and DNA of *Ehrlichia* spp. (0.7 %) was found in *H. anatolicum* ticks from the Tursunzade district. Sequence analysis of fragments of the 16S rRNA gene and *groESL* operon from *Ehrlichia* spp. revealed high similarity to *Ehrlichia* spp. The Tajik isolates of *Theileria* spp. were genotyped as *Theileria annulata* based on the analysis of 18S rRNA gene sequences. The phylogenetic analysis demonstrates that *Ehrlichia* spp. isolates are highly similar to *Ehrlichia* spp. circulating in China and Brazil. The isolate Tajikistan-5 is closely related to the putative novel species *Ehrlichia mineirensis*. The Tajik isolates of *Theileria* spp. were clustered with *T. annulata* isolates from Turkey, Iran, Pakistan, and China by phylogenetic analyses.
Key words: *Hyalomma anatolicum*; tick-borne infections; *Ehrlichia* spp.; *Theileria* spp.; Tajikistan.

For citation: Kartashov M.Yu., Kononova Yu.V., Petrova I.D., Tupota N.L., Mikryukova T.P., Ternovoi V.A., Tishkova F.H., Loktev V.B. Detection of *Ehrlichia* spp. and *Theileria* spp. in *Hyalomma anatolicum* ticks collected in Tajikistan. Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding. 2020;24(1):55-59. DOI 10.18699/VJ20.595

Выявление *Ehrlichia* spp. и *Theileria* spp. в клещах *Hyalomma anatolicum*, собранных в Таджикистане

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Аннотация. Исследовано наличие генетического материала *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp. и *Theileria* spp. в клещах *Hyalomma anatolicum*, собранных в юго-западной части Таджикистана, с последующим секвенированием и филогенетическим анализом фрагментов 16S рРНК гена и *groESL* оперона для *Ehrlichia* spp. и фрагмента гена 18S рРНК для *Theileria* spp., обнаруженного в изученных клещах. Клещи *H. anatolicum* были собраны в районах Турсунзаде и Рудаки и исследованы с использованием специфичных праймеров с помощью ПЦР на наличие генетического материала *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp. и *Theileria* spp. Выделенные ПЦР-фрагменты генов 16S рРНК, *groESL* оперона и 18S рРНК были секвенированы и проведен их филогенетический анализ с целью генотипирования обнаруженных изолятов клещевых патогенов. В клещах, собранных в районе Рудаки, обнаружена ДНК *Ehrlichia* spp. (3.3 %) и *Theileria* spp. (3.3 %), а в Турсунзаде – ДНК *Ehrlichia* spp. (0.7 %). Секвенирование фрагментов гена 16S рРНК и *groESL* оперона *Ehrlichia* spp. показало высокий уровень гомологии нуклеотидной последовательности с известными последовательностями *Ehrlichia* spp. Таджикские изоляты *Theileria* spp. были генотипированы как *Theileria annulata* на основе анализа последовательности гена 18S рРНК. Филогенетический анализ показал,

что таджикские изоляты *Ehrlichia* spp. очень близки с изолятами *Ehrlichia* spp., циркулирующими в Китае и Бразилии. Изолят Tajikistan-5 кластеризуется с предполагаемым новым видом *Ehrlichia mineirensis*. Таджикские изоляты *Theileria* spp. были генетически схожи с вариантами *T. annulata*, циркулирующими в Турции, Иране, Пакистане и Китае.
Ключевые слова: *Hyalomma anatolicum*; клещевые инфекции; *Ehrlichia* spp.; *Theileria* spp.; Таджикистан.

Introduction

Ixodid ticks transmit various pathogens to both humans and animals in Asia (Tishkova et al., 2012; Wu et al., 2013). Twenty-three species of ixodid ticks have been described in this region of Central Asia, with the predominant ixodid tick species being *Hyalomma anatolicum* Koch, 1844 (Rasulov, 2007). The Crimean-Congo hemorrhagic fever, Sindbis, and Wad Medani viruses were previously detected in ixodid ticks in Tajikistan and other Asian countries (Begum et al., 1970; Gresíkova et al., 1978; Petrova et al., 2013). *Hyalomma anatolicum* ticks are also known to transmit bacterial and parasite infections such as Lyme disease, babesiosis, piroplasmosis, theileriosis, and anaplasmosis (Tishkova et al., 2012; Wu et al., 2013). *Theileria annulata (*Piroplasmida: Family *Theileriidae*, Genus *Theileria*) is the causative agent of theileriosis in domestic animals, which is transmitted by 15 species of ixodid ticks of the genus *Hyalomma* (Robinson, 1982). *Ehrlichia* spp. (Family *Anaplasmataceae*, Genus *Ehrlichia*) are intracellular Gram-negative bacteria, ecologically associated with ixodid ticks and their animal hosts (Parola et al., 2001). The pathogenicity to domestic and wild animals, as well as to humans, has been demonstrated in *Ehrlichia canis*, *E. chaffeensis*, *E. ewingii*, *E. muris* and *E. ruminantium* (Aguiar et al., 2014; Cabezas-Cruz et al., 2014). Currently, there are no published studies on genetic markers and genotyping of *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. in *H. anatolicum* ticks in Tajikistan.

The objectives of this study were to survey the prevalence of genetic markers for these tick-borne infections in *H. anatolicum* ticks collected in southwestern Tajikistan, and to perform sequence and phylogenetic analysis of *Ehrlichia* spp. and *Theileria* spp. detected in the ticks.

Materials and methods

Tick harvesting. Adult ticks were collected from domestic animals in several villages of the Rudaki district (Somoniyon N 38°26′27″, E 68°46′28″) and the Tursunzade district (Tursunzade N 38°30′39″, E 68°13′49″) in southwestern Tajikistan in July 2009 (Fig. 1). The ticks were transported and samples for analysis were prepared as described in (Petrova et al., 2013). Tick species were identified by morphological examination with subsequent confirmation by PCR and sequencing of PCR products of a 16S rRNA fragment of the mitochondrial genome of the ticks.

PCR detection of genetic markers. DNA was isolated from tick homogenates by phenol/chloroform extraction using a commercial kit (Lytech, Moscow, Russia) following manufacturer's instructions. It was kept at −20 °C until use. The genetic markers of *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp. and *Theileria* spp. in ticks were detected by PCR with specific primers (see the Table). The PCR fragments were purified using Wizard SV Gel and a PCR Clean-Up System kit (Promega, USA) according to manufacturer's instructions. All PCR fragments were sequenced in a 3130 Genetic Analyzer automated capillary sequencer (Applied Biosystems Inc.). DNA sequencing reactions were performed with BigDyeTerminator v3.1 Cycle Sequencing Kits (Applied BioSystems, USA). Both strands of each gene fragment were directly sequenced; each sample was sequenced twice. Precautions were taken at all steps of analysis to avoid cross-contamination among samples.

Nucleotide sequences and phylogenetic analyses. DNA sequences were compared with sequences available in GenBank using the Basic Local Alignment Search Tool (BLAST) on http://blast.ncbi.nlm.nih.gov. Evolutionary analyses were conducted with MEGA5 software (Tamura et al., 2011). Multisequence alignments were performed using ClustalX. For each analyzed gene a phylogram was constructed by the maximum likelihood method. Phylogenetic distances between homologous sequences were calculated using Kimura's two-parameter model. Confidence levels for individual branches of the resulting tree were determined by bootstrap analysis with 1000 replicates.

Results and discussion

Tick harvesting

Adult *H. anatolicum* ticks (138 females and 244 males) were collected and grouped in 137 pools. Tick species were identified by sequencing a fragment of 16S rRNA mitochondrial gene for all pools. Two original variants of 16S rRNA mitochondrial gene fragment sequences found in these ticks were submitted to GenBank (accession numbers KP059123 and KP059124). The nucleotide fragments showed 99.9 % similarity to the corresponding *H. anatolicum* sequences from GenBank. These tick pools were tested by PCR for genetic markers of *Rickettsia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. and other ticks were used for genotyping. Of those ticks, 290 (179 males and 111 females)

Fig. 1. Tick collection sites in Tajikistan: *1*, Tursunzade district; *2*, Rudaki district.

Primers used for PCR identification of ticks and tick-borne infections in the present study

Species detected	Gene	Primers	Primer sequence (5′→3′)	Amplicon size, bp	Reference
Hyalomma spp. (ticks)	16S rRNA gene (mitochondrial)	H16Mf	GATTCTCATCGGTCTAAACTCAG	425	This study
		H16Mr	AGTATTTTGACTATACAAAGGTATTG		
Rickettsia spp.	*gltA*	CS409d	CCTATGGCTATTATGCTTGC	765	Roux et al., 1997
		RP1258n	ATTGCAAAAAGTACAGTGAACA		
Babesia spp./*Theileria* spp.	18S rRNA gene	BS1	GACGGTAGGGTATTGGCCT	1120	Rar et al., 2005
		BS2	ATTCACCGGATCACTCGATC		
Ehrlichia spp./*Anaplasma* spp.	16S rRNA gene	Erl1	GAACGAACGCTGGCGGCAAGC	1375	Rar et al., 2010
		Erl6	GACCCAACCTTAAATGGCTGC		
		Erl7	TAACACATGCAAGTCGAACG	1350	
		Erl8	CTTCGAGTTAAGCCAATTCC		
	groESL operon	HS1-f	CGYCAGTGGGCTGGTAATGAA	1340	Sumner et al., 1997
		HS6-r	CCWCCWGGTACWACACCTTC		

were collected in the Tursunzade district and 92 (65 males and 27 females) from the Rudaki district. The PCR tests for *Ehrlichia* spp. and *Theileria* spp. were positive in the range 0.7–3.3 %. The PCR tests for *Rickettsia* spp., *Anaplasma* spp., and *Babesia* spp. were negative in all tick samples.

Theileria identification

Theileria spp. was detected in 3.3 % ticks from Rudaki but not in ticks from Tursunzade. The amplified PCR fragments of 18S rRNA (1090–1092 bp) were isolated and sequenced (GenBank accessions KM288517–KM288519). The sequences were 100 % identical to isolates of *Theileria annulata* circulating in Turkey (AY508463) and Iran (KF429799, HM628581), similar by 99.9 % to isolated from Pakistan (JQ743630) and China (EU073963) and by 99.7 % to isolates from Spain (DQ287944). Phylogenetic analysis confirmed that *Theileria* spp. isolates from the Rudaki district of Tajikistan belonged to *Th. annulata* (Fig. 2). The analysis of 18S rRNA gene fragment for three isolates of *Th. annulata* from southwestern Tajikistan showed that all isolates were genetically identical (100 % similarity).

Ehrlichia identification

The presence of *Ehrlichia* spp. has not been previously documented in ticks and animals in Tajikistan. DNA of *Ehrlichia* spp. was detected in five pools of *H. anatolicum* ticks collected in the Rudaki and Tursunzade districts. The infection rates for *Ehrlichia* spp. were 3.3 % in Rudaki and 0.7 % in Tursunzade. The fragments of the 16S rRNA gene (1291–1352 bp) and *groESL* operon (1248–1315 bp) were sequenced (KM995818–KM995821, KP059122, KJ930191–KJ930195). The nucleotide sequences of 16S rRNA gene fragments were highly conserved (99.5–100 %) among studied isolates. The similarity levels of the studied 16S rRNA fragments to *E. chaffeensis* (CP007478), *E. canis* (KJ513197), and *E. muris* (NR121714) were 99.2, 99.2, and 99.3 %, respectively.

The phylogenetic tree generated using *Ehrlichia* spp. *groESL* operon fragment sequences was markedly different from the tree based on 16S rRNA sequences (Fig. 2, *b*, *c*). The 16S rRNA gene fragment analysis (1140 nucleotides) showed that all isolated *Ehrlichia* spp. were genetically close (see Fig. 2, *b*). The studied isolates grouped in the same branch of the phylogenetic tree as isolates from the Fujian province in Southeastern China (DQ324547) and the Tibet Autonomous Region of China (AF414399). The Tibetan isolate was grouped with the *E. canis* branch, which is genetically close to the species *E. chaffeensis*, pathogenic for humans (Wen et al., 2002). We note that the Tajik isolates were most similar to Chinese isolates from regions of China that do not border Tajikistan. The phylogenetic tree generated using *Ehrlichia* spp. *groESL* operon fragment sequences was markedly different from the tree based on 16S rRNA sequences (see Fig. 2, *c*). The nucleotide sequences of *groESL* operon *Ehrlichia* spp. found in Tajikistan are separated into three groups. Tajikistan 1 and 2 isolates were closest to two isolates *Ehrlichia* spp. from different regions of China (Xinjiang, *Hyalomma asiaticum*; Yunnan, *Rhipicephalus microplus*), Tajikistan 3 and 4 cluster with a different Chinese isolate (Xinjiang, *Hyalomma asiaticum*). Tajikistan 5 showed high similarity to *Ehrlichia* spp. (JX629806) isolated in Brazil from a *Rhipicephalus microplus* tick (Cruz et al., 2012). Tajikistan 5 has 13 nucleotide and 2 amino acid substitutions in comparison to the Brazilian isolate. The American isolate was previously identified as a new species of *Ehrlichia* spp. named *E. mineirensis*. It causes clinical manifestations associated with ehrlichiosis in experimentally infected calf (Aguiar et al., 2014).

Tajikistan 1–4 isolates clustered with Chinese isolates from Xinjiang and Yunnan Provinces. Xinjiang Province shares borders with Tajikistan in southwestern China, unlike Yunnan. Tajikistan 5 isolate was the most genetically distinct from other *Ehrlichia* spp. grouping with UFMG-EV and UFMT-BV isolates from Brazil and BOV2010 isolate from Canada (Gajadhar et al., 2010; Aguiar et al., 2014; Cabezas-Cruz et al., 2014). We infer that Tajikistan 5 isolate belongs to the putative novel species of *Ehrlichia* spp. previously named *E. mineirensis*.

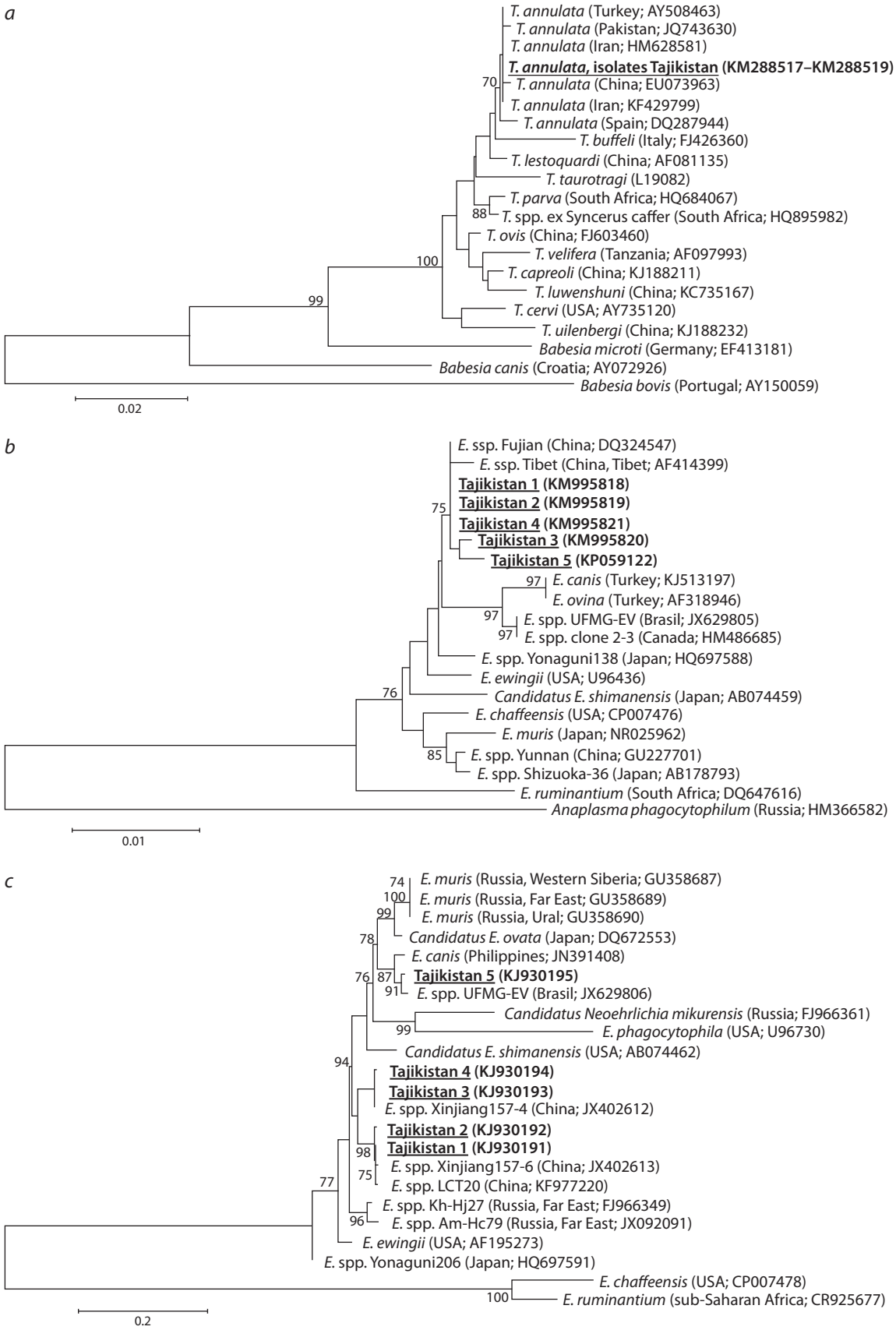

Fig. 2. Phylogenetic tree of *Theileria* spp. and *Ehrlichia* spp. isolates: *a*, based on 18S RNA of *Theileria* spp.; *b*, based on the 16S rRNA gene of *Ehrlichia* spp.; *c*, based on the *groESL* operon of *Ehrlichia* spp.

For each gene analyzed, a phylogram was constructed by the maximum likelihood method. Phylogenetic distances between homologous sequences were calculated using Kimura's two-parameter model. Confidence values for individual branches of the resulting tree were determined by bootstrap analysis with 1000 replicates.

Conclusions

Hyalomma anatolicum ticks collected in Tajikistan were tested by PCR for markers of tick-borne bacterial and protozoan infections. DNA of *Ehrlichia* spp. and *Theileria* spp. was detected in ticks collected from the Rudaki and Tursunzade districts. The infection rates for *Ehrlichia* spp. and *Theileria* spp. DNA markers ranged within 0.7–3.3 % according to PCR. Fragments of the 16S rRNA gene and *groESL* operon from *Ehrlichia* spp. and of the 18S rRNA gene from *Theileria* spp. were isolated and sequenced from *H. anatolicum* ticks. Phylogenetic analysis demonstrated that *Ehrlichia* spp. isolates were highly similar to *Ehrlichia* spp. circulating in China and Brazil. Isolate Tajikistan 5 was closely related to the putative novel species *E. mineirensis*. The Tajik isolates of *Theileria* spp. were genotyped as *Theileria annulate*, and fragments of the 18S rRNA gene from these isolates were highly similar to the 18S rRNA gene of *T. annulata* isolates from Turkey, Iran, Pakistan and China.

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Acknowledgements. This work was supported in part by the Federal Targeted Program "Scientific Potential of Higher Schools" (projects 2.1.1.7515 and 2.1.1/2743), State contract 6.657.2014/K, and the Russian Foundation for Basic Research, project 15-34-50113. The authors also acknowledge the support from the Russian Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing.

Conflict of interest. The authors of this study have no commercial associations that might create a conflict of interest to the present work. All authors are working in non-profit federal organizations. No competing financial interests exist.

Received July 5, 2019. Revised August 5, 2019. Accepted August 5, 2019.