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., 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 groEL 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 Turunzade 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 Turunzade district. Sequence analysis of fragments of the 16S rRNA gene and groEL 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.

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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 rРНК гена и groEL оперона для *Ehrlichia* spp. и фрагмента гена 18S rРНК 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 rРНК, groEL оперона и 18S rРНК 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 rРНК и groEL 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 rРНК. Филогенетический анализ показал,
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**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; Gresikova et al., 1978; Petrova et al., 2013). *Hyalomma anatolicum* ticks are also known to transmit bacterial and parasitic 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 (Párola 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* (Aguir et al., 2014; Cabezas-Cruz et al., 2014). Currently, there are no published studies on genetic markers and genotyping of *Rickettsia* spp., *Ehrlichia* spp., *Anaplasmata* 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 (Somoniyan 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., *Anaplasmata* 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., *Anaplasmata* spp., *Babesia* spp., and *Theileria* spp. and other ticks were used for genotyping. Of those ticks, 290 (179 males and 111 females)
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 *groEL* 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. *groEL* 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. *groEL* operon fragment sequences was markedly different from the tree based on 16S rRNA sequences (see Fig. 2, c). The nucleotide sequences of *groEL* 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 the studied 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*. 

### 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 (mitochondrial) | H16Mf    | GATTCTCATCGGTCTAAACTCAG                               | 425               | This study |
|                         |            | H16Mr    | AGTATTTGACTATACAAAGGTATTG                             |                   |           |
| *Rickettsia* spp.       |            | CS409d   | CCTATGCTATTGCTTGC                                    | 765               | Roux et al., 1997 |
|                         |            | RP1258n  | CTGATTCTCATCGGTCTAAACTCAG                             |                   |           |
| *Babesia* spp./*Theileria* spp. | 18S rRNA gene | BS1      | GACGGTAGGTTATTGCGCTT                                   | 1120              | Rar et al., 2005 |
|                         |            | BS2      | ATTCACCGGATCACTCGATC                                  |                   |           |
| *Ehrlichia* spp./*Anaplasma* spp. | 16S rRNA gene | Erl1     | GAACGAAGCGCTGGCCGCAAAC                                  | 1375              | Rar et al., 2010 |
|                         |            | Erl6     | GACCCACCTTTAATGGCTGC                                   |                   |           |
|                         |            | Erl7     | TAACACATGCAATGCAAG                                   | 1350              |           |
|                         |            | Erl8     | CTTCGAGTTAAGCCAAATCC                                   |                   |           |
|                         |            | groEL operon | HS1-f  | CGYCATGTTGGCTTGGTAAATGAA                                  | 1340              | Sumner et al., 1997 |
|                         |            |            |                                                      |                   |           |
|                         |            | HS6-r    | CCWCCWGTTACWACACCTTCC                                   |                   |           |
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**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 groEL 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 annulata, 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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