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A survey of ixodid tick species and molecular identification of tick-borne pathogens☆

Munir Aktas*

Department of Parasitology, Faculty of Veterinary Medicine, University of Firat, 23119, Elazig, Turkey

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ABSTRACT

This study was undertaken in two different climatic areas of Turkey to determine the presence of tick-borne pathogens of medical and veterinary importance. The ticks were removed from humans, pooled according to species and developmental stages, and analyzed by PCR, reverse line blot (RLB) and sequencing. Of the 2333 removed ticks from 10 species, 1238 (53.06%) were obtained from the arid cold zone, and the remaining 1095 (46.93%) were obtained from the humid zone. The removed ticks were identified as *Hyalomma marginatum*, *Hyalomma detritum*, *Hyalomma excavatum*, *Rhipicephalus bursa*, *Rhipicephalus turanicus*, *Rhipicephalus sanguineus*, *Dermacentor marginatus*, *Haemaphysalis punctata*, *Haemaphysalis sulcata*, *Ixodes ricinus*, *Haemaphysalis* and *Ixodes* spp. nymphs. The dominant species was *I. ricinus* (61.27%) in the humid zone, whereas the *Haemaphysalis* spp. nymph dominated (30.29%) in the arid zone. Infection rates were calculated as the maximum likelihood estimation (MLE) with 95% confidence intervals (CI). Of the 169 pools tested, 49 (28.99%) were found to be infected with the pathogens, and the overall MLE of the infection rate was calculated as 2.44% (CI 1.88–3.17). The MLE of the infection varied among tick species, ranging from 0.85% (CI 0.23–2.34) in *Haemaphysalis* spp. nymph to 17.93% (CI 6.94–37.91) in *D. marginatus*. Pathogens identified in ticks included *Theileria annulata*, *Babesia ovis*, *Babesia crassa*, *Anaplasma*/*Ehrlichia* spp., *Anaplasma ovis*, *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Hepatozoon canis* and *Hepatozoon felis*. Most tick pools were infected with a single pathogen. However, four pools infected with *H. canis* displayed infections with *B. crassa*, *A. phagocytophilum* and *E. canis*. The sequencing indicated that *Anaplasma*/*Ehrlichia* spp. was 100% identical to the sequence of *Ehrlichia* sp. Firat 2 and 3 previously identified from *Hyalomma anatolicum*.

1. Introduction

Ticks are ectoparasites of domestic and wild animals, as well as humans. They transmit a wide variety of pathogens including protozoa, bacteria, fungi and viruses that infect domestic livestock and wild animals in most regions of the world, causing diseases of zoonotic and veterinary importance (de La Fuente et al., 2008). For the development and implementation of control strategies, it is important to identify the vector ticks and their transmission pattern of the pathogens in the target geographical region.

Piroplasmosis caused by *Theileria* and *Babesia* species leads to clinical infections in domestic and wild animals with high mortality and morbidity (Friedhoff, 1997). Tropical theileriosis, caused by *Theileria annulata*, is an important tick-borne disease of cattle in tropical and sub-tropical regions (Dumanli et al., 2005). The parasite is transmitted from cattle to cattle by ticks of the genus *Hyalomma*. Ovine babesiosis, caused by *Babesia ovis*, *Babesia motasi* and *Babesia crassa*, is the most important tick-borne disease

☆ *Note*: Nucleotide sequence data reported in this paper are available in GenBank, EMBL and DDBJ databases under accession numbers from KF034779 to KF034789.

* Tel.: +90 424 237 0000; fax: +90 424 238 8173.
E-mail addresses: maktas@firat.edu.tr, vetmaktas@gmail.com

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of small ruminants (Uilenberg, 2001; Aktas et al., 2006a). Ovine babesiosis is transmitted by ixodid ticks to causing fever, anemia, hemoglobinuria and icterus in sheep and goats (Uilenberg, 2001).

Ehrlichia and *Anaplasma* species are obligate intracellular microorganisms that multiply in vertebrate reservoirs and tick vectors (Dumler, 2005). The pathogens are divided into various distinct genogroups. The genus *Anaplasma* comprises six species: *Anaplasma centrale*, *A. marginale*, *A. bovis*, *A. ovis*, *A. phagocytophilum* and *A. platys*. The genus *Ehrlichia* consists of five species, which include *Ehrlichia canis*, *E. chaffeensis*, *E. muris*, *E. ewingii* and *E. ruminantum* (Dumler, 2005). Species from both genera are transmitted by ticks of the family Ixodidae (Friedhoff, 1997).

Hepatozoon spp. are protozoan parasites that infect a wide range of domestic and wild carnivores, birds, reptiles, and amphibians and are transmitted by ingestion of ixodid ticks harboring the pathogen (Baneth, 2011). *Hepatozoon* comprises more than 300 species, 46 known to infect mammals. *Hepatozoon canis* and *Hepatozoon americanum* cause hepatozoonosis in canids. *H. canis* has long been recognized to infect and cause disease in dogs in Asia, Europe, Africa, and Latin America (Baneth and Vincent-Johnson, 2005). The main vector of the pathogen is the brown dog tick, *Rhipicephalus sanguineus*, although several tick species have been imputed to be potential vectors (Giannelli et al., 2013a).

Although tick-borne pathogens, such as, *Theileria*, *Babesia*, *Anaplasma* and *Hepatozoon* have been documented in domestic animals and tick vectors in some parts of Turkey (Altay et al., 2008; Aktas et al., 2010, 2011), there is limited information on the frequency of ixodid tick species and the prevalence of tick-borne bacteria and haemoprotozoan parasites in most areas of the country (Aktas et al., 2006b). The objective of this study was to investigate the presence of *Theileria*, *Babesia*, *Anaplasma*, *Ehrlichia* and *Hepatozoon* species in ixodid ticks from two different climatic areas of Turkey; PCR, reverse line blot (RLB) hybridization and sequencing were used for detection. Tick-transmitted pathogens are passed on either from parent to offspring tick (transovarial transmission), or ticks acquire the infection as larvae or nymphs and subsequently transmit it to the next stage (trans-stadial transmission). The infection rate for most of the pathogens was significantly higher in feeding than in questing ticks, suggesting that a number of these pathogens originated from the hosts blood ingested before tick collection rather than from transstadially maintained infections acquired during earlier blood meals. Therefore, the detection of pathogens in feeding ticks cannot establish vector competence, whereas infected unfed ticks have at least maintained the pathogen transstadially. However, additional experiments should be supported to this idea. The ticks used in this study were removed from humans a short time after they bite, not collected from vegetation.

2. Materials and methods

2.1. Study area and collection of tick samples

Tick sampling was conducted from March 2007 to December 2007 in ten provinces of Turkey: Giresun, Trabzon, Rize, Elazığ, Bingol, Mus, Malatya, Erzurum, Erzincan and Tunceli. The study area covered two climatic zones: (i) humid, in the Black Sea coastal sea region, which experiences frequent rainfall and mild temperatures (Giresun, Trabzon, Rize), the main tick species recorded being *Ixodes ricinus* (Aktas et al., 2010, 2012); and (ii) arid, in an area of eastern Turkey with warmer summers and colder winters (Elazığ, Bingol, Mus, Malatya, Erzurum, Erzincan, Tunceli), with mainly *Hyalomma* spp., *Rhipicephalus* spp., and *Haemaphysalis* spp. (Aktas et al., 2004).

At the request of The Turkish Ministry of Health, ticks recovered from humans in the provinces were sent to our laboratory (Department of Parasitology, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey). Adult and nymphal ticks were removed from patients by medical staff. The ticks were placed into 1.5 ml tubes filled with 70% ethanol or isopropanol and submitted to us along with documentation. Although the samples included immature ticks, only the adults were identified to the species level using standard taxonomic keys (Estrada-Peña et al., 2004).

A total of 2333 ticks (1667 adults and 666 nymphs) were screened for the presence of tick-borne haemoprotozoan parasites and bacteria. The ticks were washed in 70% ethanol, rinsed three times in sterile phosphate-buffered saline, and dried on filter paper. They were separated by location, species, life stage, sex, and bioclimatic zone into 169 pooled samples consisting of 143 adult (2–25 per pool) and 26 nymphal (10–44 per pool) pools and stored at −80 °C until DNA extraction. Detailed information regarding the tick samples used in this study is presented in Table 1.

The tick pools were crushed as described by Aktas et al. (2010). DNA was extracted from crushed ticks using a DNA tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. Amplification of tick-borne pathogen DNA

For the amplification of *Hepatozoon* DNA, standard polymerase chain reaction (PCR) was performed in a reaction volume of 25 μl containing 2.5 μl of the DNA sample with a pair of genus-specific primers. The forward primer HepF (5′-ATACATGAGCAAAATCTCAAC-3′) and the reverse primer HepR (5′-CTTATTATTCCATGCTGCAG-3′) were used to amplify a fragment of approximately 660 base pairs (bp) of the 18S rRNA gene of *Hepatozoon* spp., as described by Inokuma et al. (2002). Cycling conditions were as described by Aktas et al. (2013). Positive control DNA previously isolated from a dog naturally infected with *H. canis* (GenBank accession no. JQ867390) and negative control DNA (non-infected canine blood DNA and distilled water) were included in each PCR test.

The PCR for the amplification of *Babesia* and *Theileria* species was performed as previously described (Aktas et al., 2011). Genus-specific primers, RLBF2/RLBR2, were used to amplify a fragment of 460–540 base pairs (bp) of the 18S SSU rRNA gene of the V4 region of *Theileria* and *Babesia* species (Georges et al., 2001). For the identification of *Anaplasma* and *Ehrlichia* species, the primers 16S8FE and BGA1B were used to amplify a fragment of approximately 500 bp of the 16S rRNA gene of the V1 region