Journal of Equine Veterinary Science 35 (2015) 830–835

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Molecular Detection and Characterization of *Theileria equi* and *Babesia caballi* in Horses (*Equus ferus caballus*) in Turkey

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ARTICLE INFO

Article history:
Received 27 February 2015
Received in revised form 25 July 2015
Accepted 4 August 2015
Available online 8 August 2015

Keywords:
Theileria equi
Babesia caballi
Horse
Molecular characterization
Turkey

ABSTRACT

This study was carried out to evaluate the molecular prevalence of *Theileria equi* and *Babesia caballi* in horses from Southern Marmara Region of Turkey and to designate the molecular characterization of the obtained isolates. For this aim between May and July 2012, blood samples were collected from totally 203 horses. After the genomic DNA extractions from blood samples, TaqMan real-time polymerase chain reaction (PCR) analyses were performed with the specific primers which partially amplified the 18S rRNA gene region of *B. caballi* and *T. equi*. Ten (4.93%) of the examined horses were found to be infected with equine piroplasmosis (EP). The molecular prevalence of *T. equi* and *B. caballi* in the research area was determined as 2.96% and 1.97%, respectively, without a significant difference ($P > .05$). No association regarding age, gender, and breed was determined ($P > .05$) in the prevalence of EP. A total of five samples which were also positive in qPCR assay (four *T. equi* and one *B. caballi*) gave amplification on the agarose gel according to the 18S rRNA PCR assay. Thus, conventional PCR showed 66.7% and 25.0% sensitivity for *T. equi* and *B. caballi* compared with the real-time PCR. Two of four *T. equi* (BK-1 and BK-2) and one *B. caballi* (BK-3) PCR-positive isolates were sequenced for the phylogenetic analyses of their partial 18S rRNA gene sequences. BK-1 and BK-2 isolates showed 100.0% identity to each other and were determined in the *T. equi* genotype E, whereas BK-3 sequence clustered in the *B. caballi* genotype A.

1. Introduction

Equine piroplasmosis (EP) caused by apicomplexan protozoans *Theileria equi* and *Babesia caballi* (order Piroplasmida) is widespread in tropical, subtropical, and temperate areas and represents an emerging problem worldwide due to its morbidity and mortality [1]. The disease is transmitted transovarially (only *B. caballi*) and transstadially by ixodid ticks belonging to three genera, *Dermacentor*, *Rhipicephalus*, and *Hyalomma* [2]. However, iatrogenic transmission of the disease is also possible through contaminated blood transfusion, injections, and surgical instruments [3].

Clinical presentation of EP can vary from asymptomatic to severe depending on such factors as infecting dose, presence of mix infections, genetics of the parasites, and the host immune status [1]. Symptoms such as fever, anemia, intravascular hemolysis, jaundice, edema, hemoglobinuria, depression, and even death of the host occur in the acute phase of the infections [4]. Horses infected with *T. equi* usually exhibit a severe and acute form of the disease; whereas infections with *B. caballi* generally follow a chronic phase [1,4]. The horses infected with *T. equi* may remain lifelong carriers after the recovery, whereas carrier situation for *B. caballi* infections is generally limited up to 4 years [4].

Although it has a great epidemiologic interest, limited information is available on the status of EP in different kind

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0737-0806/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jevs.2015.08.002

of equines in Turkey and these studies were largely based on conventional blood smear examination of piroplasms which does not always reveal the true framework of infections. Some serological techniques such as indirect fluorescent-antibody technique (IFAT), complement fixation technique (CFT), and complement-enzyme linked immuno sorbent assay (cELISA) and limited molecular techniques based on conventional polymerase chain reaction (PCR) were also used to investigate the prevalence of EP in Turkey [5–7].

The purpose of our study is to assess the presence and molecular prevalence of *T. equi* and *B. caballi* by TaqMan-based real-time PCR assay in some horses in Bursa province of the Marmara Region in Turkey. In addition, molecular characterization and genotyping of *T. equi* and *B. caballi* from infected horses based on 18S rRNA sequences are also documented with this study for the first time in Turkey.

2. Materials and Methods

2.1. Study Area and Sample Collection

The survey was conducted from May to July 2012 in two farms, Agricultural Management Directorate and Turkish Jockey Club Karacabey stud farms which are located in the Bursa province of South Marmara Region, Turkey. Totally, 203 horses were randomly selected from the related managements, and the data on the age, sex, breed, and type of housing of the horses were recorded during the study (Table 1). Clinical signs of EP and tick infestations were also evaluated on the examined horses at the sampling time. Collected tick specimens were transferred into the sterile plastic vials and brought to the laboratory for identification. Equine piroplasmosis cases in the past and treatment for ticks in the farms were also recorded via a questionnaire. For collecting horse blood samples, the required ethical approval was obtained from the Erciyes University Animal Research Local Ethics Committee (Approval no: 09/66). The whole peripheral blood samples were collected by jugular vein puncture using vacutainer tubes with ethylenediaminetetraacetic acid (EDTA) and transported to the laboratory and subsequently stored at −20°C before further analyses.

2.2. Genomic DNA Isolation

Genomic DNA was extracted from blood samples for each horses using Multisource Genomic DNA Kit (AP-MN-MS-GDNA-250, Axygen Biosciences, USA) and eluted in 50-μL elution buffer following the manufacturer's instructions. The isolated gDNA was stored at −20°C until the molecular analysis. DNA concentrations of the samples were measured in Nano Drop Spectrophotometer (ACT Gene ASP-3700) before molecular analyses to adjust the optimum amount of gDNA used in the PCR mastermix.

2.3. TaqMan Real-Time PCR Assay

TaqMan probe-based real-time PCR assays for the detection of *T. equi* and *B. caballi* in the horse blood samples were used with the primers Be18SF, Be18SR, and the TaqMan probe Be18SP for *T. equi* and Bc-18SF402, Bc-18SR496, and TaqMan probe Bc-18SP for *B. caballi* according to the previously published assay [8] with some minor modifications on the probe dyes with the reporter 6-carboxy-fluorescein (FAM) and hexachloro-fluorescein (HEX) at the 5′ end and the quencher both black hole quencher-1 (BHQ1) at the 3′ end for *T. equi* and *B. caballi*, respectively. Amplifications were performed using Mx3005P qPCR system (Stratagene, Agilent Technologies, USA). For the duplex reaction, both TaqMan probes and the four primers were used at a final concentration of 100 nM and 250 nM each, respectively, in a total reaction volume of 20 μL comprising 1X Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies) and 50 ng template DNA. Real-time PCR amplifications were performed in duplicate using the following cycling conditions: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, and annealing at 60°C for 20 seconds.

2.4. Amplification of 18S rRNA and Phylogenetic Analysis

The genomic DNA from all blood samples was also subjected to PCR analyses with the universal "catch-all" primers TB-F and TB-R for all possible *Babesia* and *Theileria*

Table 1
Distribution and mean Ct (dR) values of *Theileria equi* and *Babesia caballi* qPCR-positive samples in relation to group of horses.

Group of Horses	Number of Individuals	Positive Individuals		Mean Ct (dR) ± Std Dev		Prevalence (%)		Total Prevalence (%)
		T. equi	*B. caballi*	*T. equi*	*B. caballi*	*T. equi*	*B. caballi*	
Age groups (y)								
0–3	68	2	2	30.59 ± 1.71	35.61 ± 2.19	2.94	2.94	5.88
4–6	31	1	1	30.85 ± 0.21	37.34 ± 0.16	3.22	3.22	6.45
>6	104	3	1	36.11 ± 2.16	38.20 ± 0.05	2.88	0.96	3.84
Gender								
Male	66	2	1	34.21 ± 4.74	37.34 ± 0.17	3.03	1.51	4.54
Female	137	4	3	32.99 ± 2.13	36.47 ± 2.15	2.92	2.19	5.11
Breed								
Arabian	152	4	3	33.74 ± 4.23	37.57 ± 0.55	2.63	1.97	4.60
English	51	2	1	32.72 ± 1.29	34.06 ± 0.05	3.92	1.96	5.88
Locality								
Agricultural Management Directorate	153	4	3	32.40 ± 3.58	36.19 ± 1.84	2.61	1.96	4.57
Turkish Jockey Club	50	2	1	35.39 ± 2.49	38.20 ± 0.14	4.00	2.00	6.00
Total	203	6	4	33.40 ± 3.37	36.69 ± 1.81	2.96	1.97	4.93

Abbreviation: Ct, threshold cycle.