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Original article

Characterization of *Theileria equi* genotypes in horses in Israel, the Palestinian Authority and Jordan

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ABSTRACT

Equine theileriosis caused by *Theileria equi* is endemic in the Middle East, where it causes a severe disease as well as widespread subclinical infection. The aim of this study was to evaluate the diversity of *T. equi* genotypes in Israel and the neighboring Palestinian Authority and Jordan. Blood samples from 355 horses from Israel, the Palestinian Authority and Jordan were tested for the prevalence of *T. equi* DNA. Two hundred and fourteen (60%) were found positive for *T. equi* infection by PCR. Of those, the 18S rRNA (1458 bp) and the EMA-1 (745 bp) genes of *T. equi* were sequenced from 15 horse samples that represent Israel's geographical distribution together with four samples from the Palestinian Authority and two from Jordan. The results were used for genotype characterization and phylogenetic analysis of *T. equi* in the equine population in Israel and its surroundings. Three 18S rRNA genotype clades were found in Israel (A, C and D) with clade D being the most prevalent and included all four isolates from the PA. In contrast, the EMA-1 gene showed little diversity with all sequences clustering in the same clade apart from one Jordanian sequence. Results suggest that although the Israeli horse population is small and relatively confined geographically, it is probable that the genetic variability, which was found among Israeli horses, is a result of introduction of horses from other countries. It also suggests that the EMA-1 gene is probably not a good target for the evaluation of variance in *T. equi* populations. Characterization of the different genotypes prevalent in a certain region is important in order to map out the intra-species sequence heterogeneity of the parasite, which is needed in order to develop new diagnostic tools and vaccines.

1. Introduction

Equine piroplasmosis (EP) is a disease of wild and domestic equids caused by the apicomplexan hemoprotozoa *Theileria equi* and *Babesia caballi* (de Waal, 1992) and transmitted by ixodid ticks of the genera *Rhiphicephalus, Hyalomma, Amblyomma*, and *Dermacentor* (Zapf and Schein, 1994; Stiller and Coan, 1995; Scoles et al., 2011). The genus *Theileria* is distinguished from the genus *Babesia* by preliminary maturation of sporozoites into schizonts within blood mononuclear cells (Mehlhorn and Schein, 1998). EP is an important disease that causes economic losses in tropical and subtropical areas and is considered a serious disease with implications for international trade whose spread is associated with the increased movement of horses all over the world (Ros-García et al., 2013). Infection with *T. equi* usually causes a severe disease producing mortality of up to 50% and becomes chronic in survivors and is considered to be life-longed if untreated. Therefore, the World Organization for Animal Health (OIE) and U.S. Department of Agriculture (USDA) implemented a mandatory screening process for international movement of horses (Hall et al., 2013). For this purpose, serological tests are available and some, such as the competitive inhibition enzyme-linked immunosorbent assay (cELISA) are considered by the OIE as the preferred test for international horse trade. Some molecular techniques developed for the detection of EP in the last decade use the small subunit ribosomal RNA (18S rRNA) gene as target due to its high conservation at the species level (Ros-García et al., 2013). However, recent findings of extensive sequence variation in the V4 hyper variable region of the 18S rRNA

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gene within *T. equi* in South Africa (Bhoora et al., 2009), and Tunisia (Ros-García et al., 2013) may account for the occasional failure to amplify the parasite DNA in serologically positive horses (Bhoora et al., 2009). This highlights the need to improve knowledge on the intra-species sequence heterogeneity, in order to be able to design diagnostic tools that detect the different genotype groups (Ros-García et al., 2013). Furthermore, genetic diversity in equine merozoite antigen (EMA-1) gene sequences of *T. equi*, reported in horses in Mongolia, should be taken into consideration when searching for relevant vaccine candidate antigens (Munkhjargal et al., 2013). Moreover, identifying the different strains of *T. equi* in an endemic region may determine if its endemicity is maintained in the population by a single strain or re-introduced with new strains from other regions. Such knowledge can contribute to the effort of monitoring the spread of the disease.

Epidemiological studies conducted in countries surrounding Israel have reported varying degrees of *T. equi* prevalence and seroprevalence in horses ranging from 9.2% and 22% in Greece (Kouam et al., 2010a) and Jordan (Qablan et al., 2013), respectively, to 35.95% in Sudan (Salim et al., 2013). Equine theileriosis is highly prevalent in Israel and the Palestinian Authority (PA) with reported seroprevalences of 50.9% and 29.6%, respectively (Aharonson-Raz et al., 2014).

The purpose of this study was to evaluate the diversity of *T. equi* in horses and perform a phylogenetic analysis of the 18S rRNA and EMA-1 genes in order to characterize different genotypes of equine theileriosis in Israel and its region.

2. Materials and methods

2.1. Blood samples

A total of 355 blood samples were collected from horses in 25 farms in Israel, nine farms in the PA and three farms in Jordan. Blood samples were collected from 273 clinically healthy horses from Israel, 66 healthy horses from the PA and 15 healthy horses from Jordan. One blood sample was collected from a horse with clinical theileriosis that was hospitalized at the Koret School of Veterinary Medicine - Veterinary Teaching Hospital and used as a positive control. Diagnosis in this horse was confirmed by PCR. Blood samples were collected from the jugular vein into EDTA tubes and kept in −80 °C until analyzed. Blood collections were performed under owners' consent and the study was approved by the Internal Ethics Review Committee of the Koret School of Veterinary Medicine, The Hebrew University (approval number: KSVM-VTH/23.2014).

2.2. DNA extraction

DNA was extracted from 50 μl of whole blood, diluted in 350 μl DDW using a commercial kit (RTP Pathogen Kit, Stratec, Germany).

2.3. PCR amplification of Theileria equi *18S ribosomal RNA gene*

The presence of *T. equi* DNA was detected using primers Bec-UF2/Equi-R (Alhassan et al., 2005) (Table 1) targeting the 18S ribosomal RNA which amplify a product of approximately 400 bp. PCR was performed in a 20 μl mixture consisting of 10 μl GoTaq® Green Master Mix (Promega, Madison, USA), 7 μl DNase/RNase-free water (Promega, Madison, USA), 1 μM of each primer and 1 μl DNA template. PCR was carried under the following conditions: an initial 5 min denaturation at 96 °C, followed by 40 cycles of 30 s at 96 °C, 45 s at 60 °C and 35 s at 72 °C, with a final 2 min extension at 72 °C.

Positive *T. equi* samples were further screened for *B. caballi* using primers Bc9_RAP2F/R (Table 1), targeting the Rap-1 gene (Bhoora et al., 2010b) and for other *Babesia* spp. using primers 5-22F,1661R (Table 1) (Birkenheuer et al., 2003) for the purpose of ruling out cross infection that might hinder sequencing of the 18S rRNA gene of *T. equi*.

Finally, the primer sets used for the purpose of sequencing the full length 18S rRNA gene were the previously tested primers NBabesia1F/18SRev-TB (Bhoora et al., 2009) (Table 1) yielding an approximately 1600 bp product. Reactions were performed in a final volume of 20 μl with High Fidelity PCR Master Mix (iProof™ High-Fidelity DNA Polymerase, BIO-RAD, California, USA) consisting of 4 μl iProof HF Buffer, 0.4 μl dNTP's Mix, 0.2 μl iProof Polymerase, 1 μM of each primer and 3 μl genomic DNA. The cycling conditions were as follows: an initial denaturation for 2 min at 98 °C, followed by 40 cycles of 10 s at 98 °C, 30 s at 60 °C and 50 s at 72 °C, with a final extension of 7 min at 72 °C. All PCR's included a non-template control (NTC), consisting of the reaction mix and 3 μl of DNase/RNase-free water (Sigma, St. Louis, MO, USA), a negative control consisting of a DNA sample from the blood of a horse positive for *B. caballi* but negative for *T. equi* (confirmed by nucleotide sequencing) and a positive control consisted of a DNA sample from the blood of a horse with clinical theileriosis and no infestation with *Babesia* spp., which was confirmed by nucleotide sequencing as well. All PCR products were purified using a PCR purification kit (MSB® Spin PCRapace, Stratec, Birkenfeld, Germany).

2.4. PCR amplification of Theileria equi *EMA-1 gene*

Detection of the EMA-1 gene was carried out using primers EMA-1F/R (Alhassan et al., 2005) (Table 1) amplifying a 750 bp product representing a fragment of the gene. Due to low levels of parasitemia, DNA was extracted from gel using a commercial kit (Invisorb spin DNA extraction Kit, Stratec, Germany) according to the manufacture's instruction and used as template for a second PCR with the same primers to augment amplification. Subsequently, DNA was extracted from gel using the same kit and sent for sequencing.

2.5. Gel electrophoresis

Gel electrophoresis was performed with 1.5% agarose in Tris–acetate–EDTA (TAE) buffer and stained with SYBR Gold (ThermoFisher Scientific, Orlando, USA) to visualize the amplified DNA fragments under UV light.

2.6. DNA sequencing

DNA sequencing was performed at Weizmann Institute of Science (Rehovot, Israel) using a 3730 DNA Analyzer (Applied Biosystems, California, USA), based on capillary electrophoresis. Sequencing of the complete 18S rRNA gene was performed using three different sets of primers following a previously published protocol (Bhoora et al., 2009). The primers NBabesia1F/18SRev-TB produce a ~1600 bp sequence. Furthermore, the PCR product was simultaneously sequenced with the internal primers: BT18S3F/R and BT18S2F/R (Matjila et al., 2008; Oosthuizen et al., 2008; Bhoora et al., 2009). Primer NBabesia1F run with primer BT18S3R produced a ~800 bp sequence at the 5′-end of the gene. Primer BT18S3F run with primer 18SRev-TB produced a ~800 bp sequence at the 3′-end of the gene while the primers BT18S2F/R produced an internal ~800 bp sequence that overlapped both the 5′ and 3′ fragments by ~400 bp.

Sequencing of a fragment of the EMA-1 gene was possible with the EMA-1F/R primers (Alhassan et al., 2005).

DNA sequences were evaluated with the ChromasPro software version 2.1.1 (Technelysium Pty Ltd., Australia) and compared for similarity with sequences available in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST, August 2016). BLAST analysis confirmed that all sequences were of the *T.*

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