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Short communication

Sequence heterogeneity in the 18S rRNA gene in *Theileria equi* from horses presented in Switzerland

Qin Liu[a,c], Marina L. Meli[b,c], Yi Zhang[a], Theres Meili[b,c], Martina Stirn[b,c], Barbara Riond[b,c], Beatrice Weibel[b,c], Regina Hofmann-Lehmann[b,c,*]

[a] *National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Key Laboratory of Parasite and Vector Biology, Ministry of Health, WHO Collaborating Center for Tropical Diseases, Shanghai 200025, China*
[b] *Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland*
[c] *Center for Clinical Studies, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland*

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ABSTRACT

A reverse line blot (RLB) hybridization assay was adapted and applied for equine blood samples collected at the animal hospital of the University of Zurich to determine the presence of piroplasms in horses in Switzerland. A total of 100 equine blood samples were included in the study. The V4 hypervariable region of the *18S rRNA* gene was amplified by polymerase chain reaction and analyzed using the RLB assay. Samples from seven horses hybridized to a *Theileria/Babesia* genus-specific and a *Theileria* genus-specific probe. Of these, two hybridized also to the *Theileria equi*-specific probe. The other five positive samples did not hybridize to any of the species-specific probes, suggesting the presence of unrecognized *Theileria* variants or genotypes. The *18S rRNA* gene of the latter five samples were sequenced and found to be closely related to *T. equi* isolated from horses in Spain (AY534822) and China (KF559357) (≥98.4% identity). Four of the seven horses that tested positive had a documented travel history (France, Italy, and Spain) or lived abroad (Hungary). The present study adds new insight into the presence and sequence heterogeneity of *T. equi* in Switzerland. The results prompt that species-specific probes must be designed in regions of the gene unique to *T. equi*. Of note, none of the seven positive horses were suspected of having *Theileria* infection at the time of presentation to the clinic. Clinicians should be aware of the possibility of equine piroplasma infections outside of endemic areas and in horses without signs of piroplasmosis.

1. Introduction

Equine piroplasmosis is an infectious, tick-borne disease caused by the hemoprotozoan parasites *Theileria equi* and *Babesia caballi*, which affect all equid species, including horses, donkeys, mules, and zebras (Wise et al., 2013). Infection with either or both of these obligate, intra-erythrocytic organisms can cause varying degrees of hemolytic anemia and systemic illness. The disease occurs throughout the tropical and subtropical areas of the world and is endemic in the areas in the middle east and southern Europe, Asia, Africa, South and Central America; it is transmitted by species of ixodid ticks of the genera *Dermacentor*, *Rhipicephalus*, *Hyalomma*, and *Haemaphysalis* (Asgarali et al., 2007; Wise et al., 2013). *T. equi* is regarded to be more widespread throughout the world than *B. caballi*. In addition, unlike *B. caballi*, *T. equi* is not completely removed from the blood after natural recovery or medical treatment. Thus, horses that recover from acute or early infection may remain as reservoirs, which can transmit the parasites to other susceptible equid species (Seo et al., 2013). So, some countries strictly restrict the import of *Theileria* or *Babesia* species serologically positive horses in horse trading (Salim et al., 2013).

The advent of molecular techniques is improving the characterization of the piroplasma species, and researchers are addressing the correct phylogenetic relationships of emerging *Theileria* and *Babesia* species (Schnittger et al., 2004). A recent serological survey of equids in Switzerland had the capacity to detect only infections with *T. equi* and *B. caballi* (Sigg et al., 2010). In contrast, using molecular tools to detect and differentiate various species and strains enables better insight into the genetic heterogeneity and diversity of equine piroplasms occurring in a given country. Thus, the objectives of the present study were to (i) detect various equine piroplasm genotypes occurring in Switzerland using the reverse

* Corresponding author at: Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.
E-mail addresses: liuqin1@chinacdc.cn (Q. Liu), mmeli@vetclinics.uzh.ch (M.L. Meli), zhang1972003@163.com (Y. Zhang), tmeili@vetclinics.uzh.ch (T. Meili), mstirn@vetclinics.uzh.ch (M. Stirn), briond@vetclinics.uzh.ch (B. Riond), bweibel@wetclinics.uzh.ch (B. Weibel), rhofmann@vetclinics.uzh.ch (R. Hofmann-Lehmann).

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line blot (RLB) hybridization assay and (ii) explore the genetic heterogeneity and diversity of these piroplasm genotypes.

2. Materials and methods

2.1. Study area and sample collection

A total of 100 equine EDTA blood samples were investigated. These samples were obtained from 90 horses from Switzerland (n = 90) and 10 horses from Germany (n = 6), Austria (n = 2), Italy (n = 1), and Hungary (n = 1) that presented at the Clinics for Equine Internal Medicine and Surgery, Vetsuisse Faculty, University of Zurich. The samples were collected for routine diagnostic purposes. The horses were presented to the clinic for various reasons between July 15 and November 23, 2009. Piroplasma infection was not considered by the clinicians as a differential diagnosis in any of the study horses. The majority of the horses (55%) had a packed cell volume (PCV) within the reference range of the laboratory (5–95 quantile: 30–42%). Overall, the median age was 12.1 years (range 0.2–39 years) for all study horses.

2.2. Nucleic acid extraction

Total nucleic acids (TNA) were extracted from 100 μL of EDTA-anticoagulated blood using a MagNaPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Rotkreuz, Switzerland). TNA was eluted in 100 μL and stored at −80 °C until use.

2.3. Reverse line blot hybridization assay

The genus-specific primers RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and RLF-R2 (5′-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3′) were used to amplify the V4 hypervariable region of the piroplasm *18S rRNA* gene, and PCR amplification was performed using touchdown PCR as described by Nijhof et al. (Nijhof et al., 2005). DNA extracted from the blood of *B. bigemina*-infected buffalo (*18S rRNA* GenBank no. KM046917) and RNase-free water were used as positive and negative controls, respectively. The blotting membrane was activated and prepared for probe hybridization as previously described (Bosman et al., 2007) and using previously described *T. equi* and *B. caballi* specific probes (Butler et al., 2008; Nagore et al., 2004).

2.4. Cloning and sequencing of the 18S rRNA gene

Five samples that hybridized only with *Babesia*/*Theileria* genus-specific and *Theileria* genus-specific probes (Table 1) were selected and the near full-length *18S rRNA* gene (approximately 1750 bp) was sequenced. For this purpose, the *18S rRNA* gene was amplified by conventional PCR using the 18S-F1 and 18S-R1 primers (Liu et al., 2005). The PCR products were cloned using the pCR4-TOPO vector and the TOPO TA Cloning Kit (Life Technologies, Zug, Switzerland) and sequencing was performed with the M13 forward and reverse primers (Microsynth, Balgach, Switzerland).

2.5. Phylogenetic and sequence analysis

The obtained sequences were submitted to GenBank under the accession numbers KM046918 to KM046922. A nucleotide query for the similarity of related organisms was conducted in NCBI using BLASTn. The sequences were aligned using the multiple sequence alignment program Clustal × 1.8 and were edited manually using BioEdit Sequence Alignment Editor (version 7.0.9.0.; Carlsbad, CA. USA). The phylogenetic trees were constructed by neighbor-joining method by MEGA6.0 (Molecular Evolutionary Genetics Analysis

Table 1
Results and characteristics of the positive horses: ID, breed, age, sex, domicile, and travel history of the horses, and laboratory parameters and analysis (packed cell volume, RLB, and sequencing).

Specimen ID[a]	Age (years)	Sex[b]	Domicile	Travel history	Reason for presentation	PCV (%)[c]	RLB results			18S rRNA cloning
							B/T[d] catchall	Theileria genus-specific	*T.equi*-specific	
S30	5	G	Waltalingen	Unknown	Colic	29	Positive	Positive		Yes
S36	24	G	Elm	Unknown	Esophageal obstruction	28	Positive	Positive		Yes
S44	20	M	Zurich	Imported from Italy 2 years ago	Transient hepatopathy (unknown origin)	37	Positive	Positive		Yes
S57	18	M	Bubikon	Visit to France	Colic, euthanasia	29	Positive	Positive		Yes
S82	6	S	Hedingen	Unknown	Dental care	30	Positive	Positive	Positive	No*
S96	10	S	Remingen	Imported from Spain 7 years ago	Coughing, suspected Dictyocaulus arnfieldi infection	46	Positive	Positive	Positive	No
H97	13	S	Budapest	Domicile Hungary	Osteosynthesis, euthanasia	40	Positive	Positive		Yes

[a] S: Switzerland; H: Hungary.
[b] G: gelding; M: mare; S: stallion.
[c] PCV: packed cell volume; reference range 5–95 quantile: 30–42%.
[d] B/T *Babesia/Theileria* genus specific probe.
* Failed to yield visible bands of the full-length *18S rRNA* gene by PCR but a 400-bp band was obtained by the RLB-F2 and RLB-R2 primers, which was subsequently cloned and sequenced.