



Short communication

Sequence heterogeneity in the 18S rRNA gene in *Theileria equi* from horses presented in 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) ($\geq 98.4\%$ 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.

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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 *Haema-*

physalis (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

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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 RLB-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		Theileria genus-specific	<i>T. equi</i> -specific	18S rRNA cloning
							B/T ^d	catchall			
S30	5	G	Waltalingen	Unknown	Colic	29	Positive	Positive	Positive		Yes
S36	24	G	Elm	Unknown	Esophageal obstruction	28	Positive	Positive	Positive		Yes
S44	20	M	Zurich	Imported from Italy 2 years ago	Transient hepatopathy (unknown origin)	37	Positive	Positive	Positive		Yes
S57	18	M	Bubikon	Visit to France	Colic, euthanasia	29	Positive	Positive	Positive	Positive	Yes
S82	6	S	Hedingen	Unknown	Dental care	30	Positive	Positive	Positive	Positive	No
S96	10	S	Remingen	Imported from Spain 7 years ago	Coughing, suspected Dictyocaulus imfieldi infection	46	Positive	Positive	Positive	Positive	No
H97	13	S	Budapest	Domicile Hungary	Osteosynthesis, euthanasia	40	Positive	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.

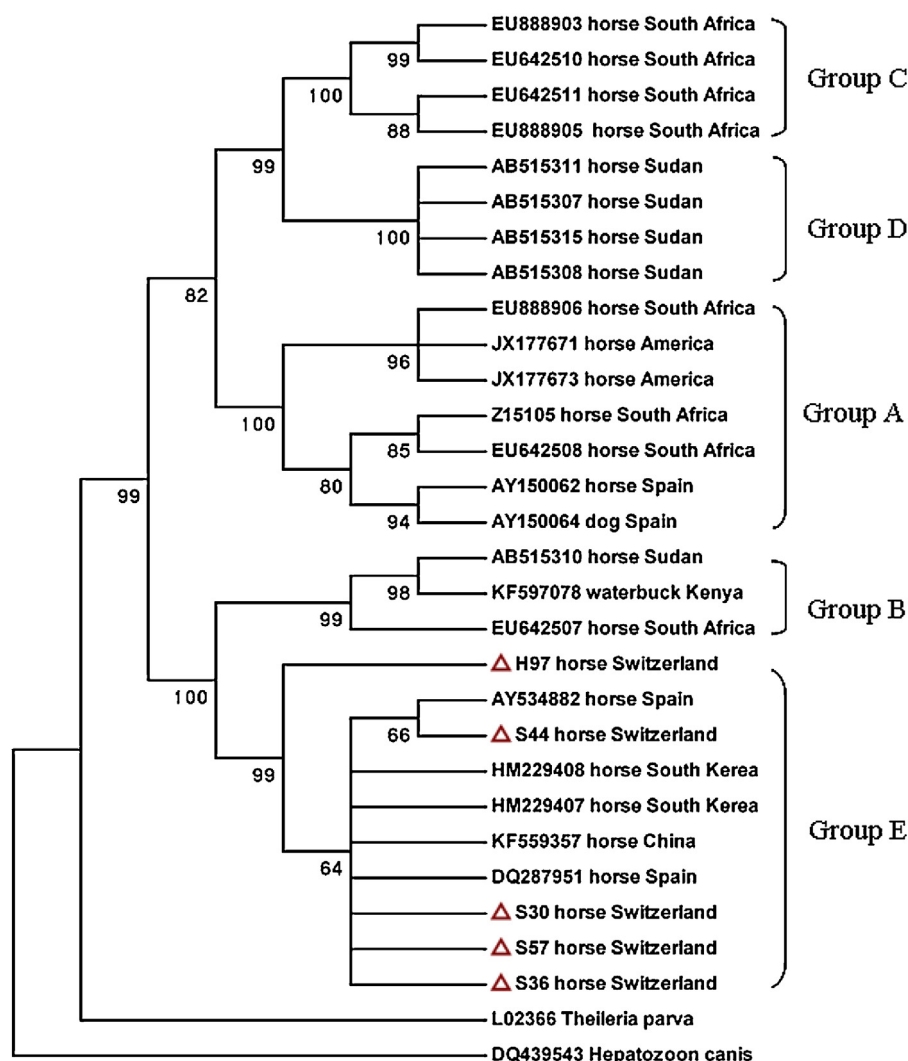


Fig. 1. Rooted phylogenetic tree showing the relationship of the identified *T. equi* genotypes and species. The relationship was determined using the 18S rRNA gene sequences by neighbor-joining with 1000 bootstrap resamplings. *T. equi* samples identified in this study (S30, S36, S44, S57, and H97) are indicated by the symbol "Δ". Other *T. equi* from GenBank are shown by their accession numbers, host, and country. Scale bar units are nucleotide substitutions per position. The *T. equi* 18S rRNA gene sequences fell into five main groups, designated as Groups A, B, C, E and D, as previously described (Qablan et al., 2013).

Version 6.0) (Tamura et al., 2013). Distance and maximum likelihood models were applied using 1000 bootstrap replicates per tree for each method. The 18S rRNA gene sequence of *T. parva* (L02366) and *Hepatozoon canis* (DQ439543) were included in the trees as outgroups. All of the generated consensus trees were edited using the MEGA 6.0 software package (Tamura et al., 2013).

The pairwise nucleotide percent identity of new sequences with a previously published sequence profile of related organisms was calculated using MegAlign software (DNASTar Inc., Madison, WI, USA). The evolutionary divergence between new sequences of the present study and previous sequences of related organisms based on pairwise base difference was estimated by completely deleting positions that contained gaps and missing data using the MEGA 6.0 software package (Tamura et al., 2013).

3. Results

3.1. Prevalence and RLB results

RLB hybridization revealed that 7% (7/100) of the samples hybridized with the *Babesia/Theileria* genus-specific and *Theileria* genus-specific probes. Moreover, of the seven positive samples, two

samples produced a signal with the *T. equi*-specific probe (Table 1). When looking at the origin of the horses, six of the 90 horses residing in Switzerland (6.7%) were positive for the *Babesia/Theileria* genus-specific and the *Theileria* genus-specific probe and among them two (2.22%) were positive for the *T. equi* probe (Table 1). Of the 10 horses from abroad, one (10%) tested positive for the *Babesia/Theileria* genus-specific and *Theileria* genus-specific probe but not the *Theileria* species-specific probe. This horse (H97) was from Hungary (Table 1). Of the six horses from Switzerland that tested positive, one horse (S57) was recently in pasture in France (twice, for 2 and 4.5 months), and two horses (S44, S96) were imported from Italy 2 years previously and from Spain 7 years previously, respectively (Table 1). The three other horses had not been abroad to the owners' knowledge. Selected characteristics of the positive horses are listed in Table 1. Two of the infected horses (S44, S57) had a history of weight loss, and one (S44) was reported to show intermittent icteric mucous membranes without other clinical signs. At the time of blood collection, three of the infected horses were slightly anemic (Table 1).

Z15105	TTTCT GCTGTTTCGT TGA-CTGCGC T-TGGCGTTT G-TCATCGTT GCGGCTTGGT TGGGT	(A ²)	A ¹
EU6424508	TTTCT GCTGTTTCGT TGA-CTGCGC T-TGGCGTTT G-TCATCGTT GCGGCTTGGT TGGGT	(A)	
AY150062	TTTCT GCTGTTTCGT TGA-CTGCGC T-TGGCGTTT G-TCATCGTT GCGGCTTGGT TGGGT	(A)	
JX177671	TTTCT GCTGTTTCGT TGA-CTGCGT T-TGGCGTTT G-TCATCGTT GCGGCTTGGT TGGGT	(A)	
EU888906	TTTCT GCTGTTTCGT TGA-CTGCGT T-TGGCGTTT G-TCATCGTT GCGGCTTGGT TGGGT	(A)	
EU888903	TTTCT GCTGTATCGT TATCTTCTGC T-TGACAGTT TGATTTCGTT ATGGCTTAGT TGGGG	(C)	B
EU888905	TTTCT GCTGTATCGT TATCTTCTGC T-TGACAGTT TGGTATCGTT ATGGCTT-GT TGGGT	(C)	
EU642510	TTTCT GCTGTATCGT TATCTTCTGC T-TGACAGTT TGATTTCGTT ATGGCTTAGT TGGGG	(C)	
EU642511	TTTCT GCTGTATCGT TATCTTCTGC T-TGACAGTT TGGTATCGTT ATGGCTT-GT TGGGT	(C)	
AB515307	TTTCT GCTGTATCGT TTTCTTCTGC T-TGACAGTT GGATTTCGTT ATGGCTTAGT TGGGT	(D)	
S30	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	C
S36	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	
S44	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	
S57	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	
H97	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	
AY534882	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	
DQ287951	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	
KF559357	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	
HM229407	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GATCTTGTTC GTGGCTTAGT TGGGG	(E)	
KF597078	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GGGCCTCGTT GTGGCTTAGT TGGGG	(E)	
AB515310	TTTCT GCTGCATCGT GGTTCTTCGC TATGTCGAGT GGGCCTCGTT GTGGCTTAGT TGGGG	(D)	
EU642507	TTTCT GCTGCATCGT GGTCCTTCGC TATGTCGAGT GGTCCCTCGTT GTGGCTTAGT TGGGG	(B)	

-----520-----530-----540-----550-----560-----570-----

Fig. 2. Nucleotide sequence alignment. A 60-bp region of the V4 hypervariable region of the *18S rRNA* gene from variants identified in this study (S30, S36, S44, S57, and H97) and previously published *T. equi* (the Genbank accession numbers are listed in the figure). (1) The sequences are grouped into the three groups, A, B and C, in accordance with an earlier publication (Bhoora et al., 2009). The grey box indicates the RLB probe regions variance; the sequence of the *T. equi* RLB probe used in the present study is underlined (Nagore et al., 2004). Nucleotides that differ from the probe sequence are indicated in bold. (2) The letters in parenthesis behind the sequences (A to E) indicate to which group the sequence belongs according the classification used in Fig. 1 (Qablan et al., 2013).

3.2. Nucleotide sequence heterogeneity analysis

Five samples (S30, S36, S44, S57, and H97; Table 1) hybridized only with *Theileria* genus-specific probes but not with any of the *Theileria* species-specific probes. The *18S rRNA* gene from these five samples were cloned and sequenced. Blast analysis revealed that the *18S rRNA* gene sequences of the five samples were related to the *T. equi* sequence and had 97.9% to 99.5% identity among each other with a nucleotide difference of 8–36 bp. Interestingly, the identity for H97 (originating from the horse living in Hungary) was lower ($\leq 98.2\%$) than that for the four other new sequences ($\geq 99.2\%$). Blast analysis revealed that the new *18S rRNA* gene sequences were closest to *T. equi* isolated from a horse in China (GenBank accession number: KF559357) and from a horse in Spain (GenBank accession number: AY534882), with identities of 98.4% to 99.9% and 98.4% to 99.7%, respectively, and nucleotide differences of 4–27 bp and 10–27 bp, respectively. No *Babesia* spp. were detected in the present study.

Phylogenetic analyses of the near full-length *18S rRNA* gene of *T. equi* sequences demonstrated five main groups, designated as Groups A, B, C, E and D (Fig. 1), as previously described (Qablan et al., 2013). The five new sequences (S30, S36, S44, S57, and H97) that hybridized only with *Theileria* genus-specific probes but not with any of the *Theileria* species-specific probes fell all into Group E (Fig. 1). Within group E, the new sequences, S30, S36, S44, and S57 appeared in the same clade, and H97 formed a distinct

monophyletic group (Fig. 1). Two samples (S82 and S96; Table 1) hybridized with the *T. equi* RLB probe used in the present study (Nagore et al., 2004). An attempt to sequence the full-length *18S rRNA* gene of S82 failed, but an approximately 400 bp fragment was obtained using the RLB-F2 and RLB-R2 primers. When using this short PCR product for phylogenetic analysis, S82 fell into Group A of the five main groups previously described (Qablan et al., 2013); the 400 bp fragment of S82 was 100% identical to the partial sequence of Z15105 (data not shown).

Sequence alignment of the V4 hypervariable region, to which the *T. equi*-specific probe was designed revealed that all of the *18S rRNA* gene sequences from variants that failed to hybridize with the *T. equi*-specific probe had sequence differences in this region (Fig. 2). The sequences of the five samples (S30, S36, S44, S57, and H97) differed from the probe sequence by 7 bp (Fig. 2).

4. Discussion

The RLB assay allows for the simultaneous detection and differentiation of many piroplasm species and permits strain or genotype identification of the piroplasms present (Bhoora et al., 2009; He et al., 2012). Various undescribed piroplasm species were identified using this approach (Chaisi et al., 2011; He et al., 2012). The present study is the first to report the use of the RLB assay for the genotype identification of equine piroplasms in Switzerland.

The 18S rRNA gene is widely used as a genetic marker and for phylogenetic analyses of piroplasms due to its low substitution rate, constrained and conserved function, and occurrence in multiple copies (Hunfeld et al., 2008). Two genotypes were originally proposed for the *T. equi* species (Nagore et al., 2004), but later studies from South Africa (Bhoora et al., 2009), Sudan (Salim et al., 2010), Jordan (Qablan et al., 2013) and American (Hall et al., 2013) have added one to three additional genotypes. The intraspecific diversity of *T. equi* is likely to increase with the addition of sequences from currently unexplored geographical regions.

Based on the results from the RLB assay and 18S rRNA gene sequences, we identified two genetically distinct *T. equi* genotypes. One of these was recognized by a *T. equi*-specific probe reported earlier (Butler et al., 2008); this genotype was first described in South Africa (Allsopp et al., 1994). According to a recent classification, this genotype was designated Group A (Qablan et al., 2013; Fig. 1). The other genotype we found differed by 7 bp in the probe region and was recognized, in the aforementioned classification, as Group E (Qablan et al., 2013; Fig. 1). Our results show that *T. equi* exhibits an overlapping occurrence of different genotypes within the same population of equids, which is in accordance with previous reports (Bhoora et al., 2009; Qablan et al., 2013; Salim et al., 2013). Of note, the sequence of H97 originating from a horse in Hungary was more distantly related to the other species and only shared 97.9% to 98.2% similarity with the other species. Whether this finding indicates that H97 may represent a novel variant would require further confirmation.

In the present study, the analysis of the 18S rRNA gene variants in the short region of the probe binding area revealed three main genotype groups (Groups A, B, and C) (Fig. 2), with differences of approximately 7–10 bp between the groups. This was consistent with a previous publication (Bhoora et al., 2009). Group A contained the *T. equi* sequence first reported in South Africa (accession number: Z15105) and the probe from South Africa (Allsopp et al., 1994); this was also the *T. equi*-specific probe used in our study, and our samples S82 and S96 hybridized with it. Group B contained the *T. equi* sequences first reported in South Africa (accession numbers: EU642510, EU642511, EU888903, and EU888905) (Bhoora et al., 2009) and also in Sudan (accession numbers: AB515307) (Salim et al., 2010). Group C was a novel group and contained sequences first reported in Spain (accession number: AY534882) (Nagore et al., 2004) and many newer sequences reported from China (accession number: KF559357) (Tian et al., 2013), Kenya (accession number: KF597078) (Githaka et al., 2014), and Sudan (accession number: AB515320) (Salim et al., 2010), as well as the five new Swiss *T. equi* 18S rRNA sequences from our study (S30, S36, S44, S57, and H97) that only hybridized with the *Babesia/Theileria* catchall and *Theileria*-specific probes.

The molecular prevalence of *Theileria* infection in equids in the present study was 7%, which is in agreement with the study by Sigg et al., who used standard serological tests in Swiss horses in 2010 and found 7.3% seropositivity for equine piroplasma (Sigg et al., 2010). No *Babesia* spp. were detected in the present study. Moreover, no significant differences in the prevalence of *Theileria* infections were observed when the samples were grouped according to horse origin (Switzerland versus abroad), gender, and age. However, these analyses may have been hampered by the limited number of samples tested and biased sample population (all of the horses were presented to the clinic in Zurich). Although some of the *Theileria*-positive horses in this study showed mild anemia, weight loss, or transient icteric mucous membranes, they were considered to be asymptomatic carriers at the time of presentation. It is well known that *T. equi* persists as a lifelong infection. Although asymptomatic horses have low parasitemia, the transmission of *T. equi* can still occur either iatrogenically or when competent tick vectors feed on these horses (Ueti et al., 2008). Asymptomatic and persis-

tently infected carriers can serve as reservoirs of infection, which is a big challenge in controlling the spread of this parasite. It may be advisable that horses in Switzerland be examined for piroplasm infections, especially when imported from or returning home after travelling abroad. Clinicians should be aware of the possibility of equine piroplasma infections outside of endemic areas.

5. Conclusion

The RLB hybridization assay allows for the simultaneous detection of piroplasm species and adding 18S rRNA gene sequences analysis can permit to differentiate or identify different strains or genotypes of piroplasms. In this study, two different *T. equi* genotypes were detected in the horses presented to the Clinics for Equine Internal Medicine and Surgery, at the University of Zurich. The horses did not show obvious signs of piroplasmosis; thus, clinicians should be aware of the possibility of equine piroplasma infections in healthy horses or horses presented for unrelated medical reasons.

Conflict of interests

The authors declare no competing interests.

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