Parasite load and genotype are associated with clinical outcome of piroplasm infected equines

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Research

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Abstract

Background: Equine piroplasmosis (EP) is a highly endemic protozoan disease of horses worldwide. While most horses in endemic areas are subclinically infected, the mechanisms leading to clinical outcome are vastly unknown. Moreover, since clinical signs of disease are not specific, and the prevalence in endemic areas is high, it is difficult to determine if EP is the cause of disease. To identify possible mechanisms leading to the clinical outcome in an endemic area, we compared parasite loads and genotypes in clinically and subclinically infected horses.

Methods: Blood was collected from horses with clinical signs consistent with EP, and from apparently healthy horses from various locations. Packed cell volume (PCV) and total solids (TS) were measured from each sample. Quantitative and diagnostic PCR were used to quantify and classify EP infection in clinical and subclinical horses.

Results: For both parasites, clinical cases were associated with low mean packed cell volume (PCV) and high mean parasite load ($P<0.001$), enabling to determine a cutoff value between clinically and subclinically infected horses. *Theileria equi* of subclinical horses classified into three different 18S rRNA genotypes: D (=23), A (n=12) and C (n=5), while all clinical cases classified as genotype A. The sequences of *T. equi* equi merozoite antigen-1 (ema-1) gene were fairly conserved and all classified as genotype A. The *ema-2* sequences were analyzed with all currently published sequences and were mostly classified into one genotype (A) among the three resolved genotypes. *Babesia caballi* rhoptry associated protein-1 (rap-1) was classified into sub-genotypes A1 (n=14) and A2 (n=5) with no association to clinical outcome. Classification of the 18S rRNA gene (sub-genotypes B1 and B2) was in agreement with the *rap-1* classification.

Conclusions: The results of this study suggest that quantification of parasite loads of infected horses may be used to distinguish between clinical and subclinical cases. Additionally, we identified *T. equi* 18S rRNA genotype A to be associated with clinical disease. This finding emphasizes the importance of in-depth genetic characterization of *T. equi* genotypes to identify possible markers for virulence.

Background

Equine piroplasmosis (EP) is an important tick-borne disease of equids, caused by the hemoprotozoan apicomplexan parasites Theileria equi and Babesia caballi. Both parasites are endemic in most parts of the world including South America, Africa and most parts of Asia and Europe [1, 2]. Introduction of these parasites to non-endemic areas suitable for tick vectors may cause epizootic spread of disease [1, 3]. Theileria equi is usually more prevalent than B. caballi in endemic regions, has more severe clinical manifestation and leads to life-long infestation if untreated [1, 2].

Clinical disease is usually characterized by acute hemolytic anemia, and ranges from subclinical, non-apparent carriage to peracute, life-threatening disease. Clinical signs of acute disease include anemia, jaundice, inappetence, edema and pigmenturia. Most infected horses become a-symptomatic carriers

after resolution of clinical signs [1, 2]. The factors contributing to the development of clinical disease are unclear. Innate immunity plays a central role in immune response, while adaptive immunity is also essential and high antibody titer correlates with parasite control [1, 4]. Early exposure in endemic areas usually leads to protective immunity, while primary exposure of naïve adults more often leads to clinical disease. However, clinical cases in adult horses are also reported in endemic areas.

Apicomplexan parasites, including T. equi and B. caballi, show genetic diversity [5–8]. Five T. equi and two B. caballi 18S rRNA genotypes were described [9, 10], and none was associated with parasite virulence. In recent years 18S rRNA based classification has been questioned, as evidences for considerable genetic variation in other loci led to the description of several potential new species [6, 7]. The novel species T. haneyi has 18S rRNA sequence similar to genotype C but has a much smaller and considerably different genome than T. equi [7], and three variants of T. africa has 18S rRNA sequences similar to genotype D, but differs from T. equi in its 30S rRNA gene sequence [6].

In order to detect genetic variation that is linked to parasite evasion of the host immune system, known immunogenic proteins were characterized: in B. caballi, rhoptry associated protein-1 (rap-1) gene and protein [11–13], and in T. equi, equi merozoite antigen (ema) gene family, which includes nine genes [10, 14–16]. Two B. caballi rap-1 genotypes have been characterized with considerable differences in protein structure [11], jeopardizing the results of the United States Department of Agriculture (USDA)-approved cELISA detection kit (based on the RAP-1 protein) on isolates from Africa and the Middle East [11–13]. The T. equi ema-1 and ema-2 genes and proteins are more conserved, and were used for the development of serological assays [17, 18]. Some sequence variation has been detected for these genes, although little diversity is found among isolates within a geographical region [10, 14, 19].

The aim of this study was to identify possible mechanisms leading to the clinical outcome of T. equi and B. caballi infection in an endemic area, by comparing clinical and subclinical cases. The use of quantitative tools and additional parasite gene sequences was applied to overcome the limitations of classification based solely on the 18S rRNA gene.

Methods

Sample collection

Blood samples from horses exhibiting clinical signs associated with EP were collected by their attending veterinarians and sent to the laboratory for molecular diagnosis, along with results of packed cell volume (PCV), when available (N = 25). Additionally, two samples were collected from horses with non-specific or unrelated clinical signs, and on which tick infestation was documented at the time of examination.

Blood samples from subclinical horses were collected as a part of a surveillance study of sentinel farms designed to represent the distribution of the horse population in Israel. Blood from all horses was collected from the jugular vein into sterile vacuum tubes containing Ethylenediaminetetraacetic acid

(EDTA). Packed cell volume (PCV) and total solids (TS) were measured using standard methodology prior to storage at -20 °C until processing.

The characteristics of all horses included in this study are listed in Table S1.

DNA extraction

DNA was extracted from 50 µl of whole blood of each sample, diluted in 350 µl double distilled water (DDW) using a commercial kit (RTP Pathogen Kit, Stratec, Germany), according to the manufacturer's instructions.

Identification of EP parasites using polymerase chain reaction (PCR)

Initial screening for infestation with EP parasites was performed using diagnostic PCR directed to identify a 400 bp fragment of T. equi 18S rRNA gene [20], and B. caballi rap-1 gene [11], as previously described [10, 12, 21] (For primer list, see Table 1).

Quantification of EP parasitemia using quantitative real-time PCR reaction (qPCR)

Quantification of parasitemia was assessed via qPCR using TaqMan minor groove binder (MGB™) probes targeting T. equi ema-1 gene [14] and B. caballi 18S rRNA gene [22] (Table 1). A clean PCR product of each gene was used to prepare the standard curve, and gene copy number (gcn) was calculated from the molecular weight and gene length [gcn = (ng x gcn/mole) / (bp x ng/g x g/mole of bp)]. A standard curve of 1-10^8 copies was used to determine copy number in each sample. The cutoff for parasite detection was set as one copy. The standard curve of each parasite was later compared with DNA extracted from blood with a known percentage of parasitized erythrocytes (obtained from culture diluted in non-infected horse blood) to extrapolate from gene copies to infected red blood cells [23].

Parasite quantities of clinical versus subclinical cases and of T. equi versus B. caballi subclinical cases were compared using Mann-Whitney nonparametric statistical analysis. Receiver operating characteristic (ROC) curves were generated to establish cutoff values to differentiate parasitemia between clinical and subclinical cases of each parasite. The statistical analysis was performed in SPSS 22.0® software.

Amplification and sequencing of T. equi and B. caballi 18S rRNA gene

The full length (1600 bp) of T. equi 18S rRNA gene was amplified using primers NBabesia1F and 18SRev-TB primers [5, 24, 25] (Table 1), as previously described [10]. All positive PCR products were cleaned using Exonuclease I and Shrimp alkaline phosphatase (New England Biolabs Inc., Massachusetts, US) and sent for sequencing (Macrogen Europe, Amsterdam, The Netherlands). Sequencing of the complete 18S rRNA

gene was performed using three different sets of primers, as described [5, 10] (Table 1). Full length sequences were amplified from all available clinical samples and B. caballi-positive samples, and from 50 T. equi-positive subclinical samples, five horses per positive farm.

Amplification and sequencing of T. equi equine merozoite antigen 1 and 2 (ema-1 and ema-2) genes

A 750 bp fragment of T. equi ema-1 gene was amplified using primers EMA-1F/R [20] (Table 1). An 800 bp fragment of T. equi ema-2 gene was amplified using primers EMA-2F/R (Kumar el at., 2018) (Table 1). All positive PCR products were cleaned using Exonuclease I and Shrimp alkaline phosphatase (New England Biolabs Inc., Massachusetts, US) and sent for sequencing in Macrogen Europe (Amsterdam, The Netherlands) using both the forward and reverse primers.

Amplification and sequencing of B. caballi rhoptry-associated protein-1 (rap-1) gene

A fragment of approximately 1500 bp fragment of B. caballi rap-1 gene was amplified using the primers Bc9_RAPF/R, as previously described [11, 12] (Table 1). All positive PCR products were cleaned using Exonuclease I and Shrimp alkaline phosphatase (New England Biolabs Inc., Massachusetts, US) and sent for sequencing in Macrogen Europe (Amsterdam, The Netherlands) using both the forward and reverse primers as well as the internal set of primers Bc9_RAP2F/R (Table 1), as previously described [11, 12].

Phylogenetic analysis

Sequences of each sample, gene and primer were evaluated using the Chromas software version 2.6 (Technelysium Pty Ltd., Australia), and a consensus sequence for each sample and gene was constructed using the MEGA 7.0.18 software [26] (http://www.megasoftware.net, November 2018), by multiple sequence alignment of sequence results of all primers. BLAST (http://www.ncbi.nlm.nih.gov/BLAST, Last accessed November 2019) analysis confirmed that all constructed sequences were 99–100% identical to previously published sequences of the corresponding gene available in the GenBank. The constructed sequences from all samples and genes were submitted to the GenBank.

The constructed sequences of all samples of each gene were compared using multiple sequence alignment (ClustalW) using the MEGA7 software, to identify gene variants between samples. Additional sequences available in the GenBank and representing all previously characterized genotypes of each gene were added to the analysis, in addition to several sequences of other apicomplexan parasites and sequences of other genes of the ema family, in the analyses of ema-1 and ema-2. Sequences were aligned using the MEGA7 software, using the MUSCLE function [27], and trimmed on both ends to receive a comparable sequence.

Phylogenetic trees were constructed using both Maximum Likelihood and Neighbor Joining methods in MEGA7. The results obtain by both methods were similar for all analyses. The statistical model used for each analysis was selected by the lowest Bayesian Information Criterion (BIC) score, and an acceptable Akaike Information Criterion (AIC) score, calculated in MEGA7.

The model selected for the analysis of 1079 positions of the T. equi 18S rRNA gene Tamura-Nei + G [28], while the model selected for the analysis of 1212 positions of B. caballi 18 rRNA gene was Tamura-Nei + G + I [28]. The model selected for the analysis of 400 positions of T. equi ema-1, 782 positions of T. equi ema-2 and 251 positions of B. caballi rap-1 genes was Kimura 2-parameter + I [29]. The model selected for analysis of a longer segment of 1186 positions of B. caballi rap-1 was Kimura 2-parameter + G [29]. All algorithms were constructed with bootstrap replicates from 1000 randomly selected samples to estimate reliability.

The divergence between T. equi ema-2 sequences from this study and from GenBank was estimated using distance matrix analysis, Kimura 2-parameter + G model in MEGA7.

Results

Study population

Blood samples were collected from 13 horses exhibiting clinical signs consistent with EP including fever, anemia, icterus and inappetence. In seven of these cases parasites were identified in bloodsmear (others were not tested). Diagnostic PCR identified T. equi infestation in seven cases and B. caballi infestation in six out of the 13 clinical cases. One of the T. equi cases was a fatal case of a neonatal filly born to a subclinically infected mare [30]. All clinical cases were from the north or center of Israel. Ages ranged between newborn and 17 years. Sex, breed, mean age and mean packed cell volume (PCV, was available in ten cases) are listed in Table 2.

Samples from subclinical horses were collected from apparently health horses and from two horses with heavy tick infestation, but with no clinical signs characteristic of EP. Forty T. equi positive and 13 B. caballi positive samples were included in the analyses. All subclinical cases were also from the north or center of Israel. Ages ranged between two and 22 years. Sex, breed, mean age and mean PCV (available in 59 cases) are listed in Table 2.

The mean PCV of clinically infected horses was significantly lower than of subclinically infected horses ($P < 0.001$ for both parasites).

Quantification of EP parasitemia in clinical versus subclinical horses

Quantitative real time PCR was used to evaluate T. equi parasitemia in six clinically infected and seven, randomly selected, subclinically infected horses of which one had high tick infestation and unrelated

clinical signs. Parasitemia of clinically infected animals ranged between 1,998 and 84,270 ema-1 gene copies (mean: 31,573.1, SEM: 13,370.4), equivalent to 0.12–5.3% parasitized erythrocytes (PE), while in subclinical animals it ranged between 5 and 81 gene copies (mean: 27.3 SEM: 11.2), equivalent to 0.0003–0.005% PE. The horse that was heavily infested with ticks had the highest parasite load in the subclinical group (Fig. 1A). The difference between parasite loads of clinical versus subclinical animals was statistically significant (Mann-Whitney, P = 0.001). A diagnostic cutoff was calculated as 1040 gene copies or 0.066% PE with a sensitivity and specificity of 100% (ROC AUC = 1, P = 0.003).

Quantitative real time PCR was used to evaluate B. caballi parasitemia in six clinically infected and seven, randomly selected, subclinically infected horses, one of the latter with high tick infestation and unrelated clinical signs. Parasitemia of clinically infected animals ranged between 503 and 152,696 18S rRNA gene copies (mean: 38,348, SEM: 24,587.7), equivalent to 0.007–2.11% PE, while in subclinical animals it ranged between 10 and 88 gene copies (mean: 40.5 SEM: 10.9), equivalent to 0.0001–0.0012% PE. The horse that was heavily infested with ticks had the highest parasite load in the subclinical group (Fig. 1B). The difference between parasite loads of clinical versus subclinical animals was statistically significant (P = 0.001). A diagnostic cutoff was calculated as 296 gene copies or 0.004% PE with a sensitivity and specificity of 100% (ROC AUC = 1, P = 0.003).

Classification of T. equi and B. caballi based on 18S rRNA gene

The 18S rRNA gene was successfully amplified and sequenced in all T. equi clinical and subclinical samples (MK392050-MK392061, MN611313-MN611352) and from all B. caballi clinical samples (MK288106-MK288110, MN629354). All B. caballi-positive subclinical horses were co-infected with T. equi and, therefore, could not have been classified based on their 18S rRNA gene.

Out of the five T. equi 18S rRNA genotypes (A-E) [5, 31], all seven clinical samples and 12 (30%) subclinical samples were classified as genotype A; 23 (57.5%) subclinical isolates were classified as genotype D, and the remaining five (12.5%) subclinical isolates were classified as genotype C (Fig. 2).

Of the two B. caballi 18S rRNA genotypes (A and B, with genotype B subdivided into subgroups B1 and B2 [5], the six clinical samples were classified as genotype B: Four samples as genotype B1 and two samples as genotype B2 (Fig. 3).

Classification of T. equi based on ema-1 and ema-2 genes

Five Theileria equi ema-1 PCR products were successfully amplified and sequenced from clinical and five subclinical horses (MK415929-MK415937). Out of the three T. equi ema-1 genotypes (A-C [14], all samples were classified as genotype A (Fig. 4).

Theileria equi ema-2 PCR products were successfully amplified and sequenced from all seven clinical and five subclinical horses (MN624965-MN624979). The 12 sequences obtained in this study were analyzed with all available T. equi ema-2 sequences in the GenBank (20). Based on mean evolutionary distance (Table 3), three genotypes were classified (A-C). The mean evolutionary distance within each genotype was under 0.004 base substitutions, and the mean difference between groups was 0.011–0.059 base substitutions. All clinical and three subclinical isolates were classified as genotype A, and two subclinical isolates were classified as genotype C (Fig. 5).

Of the ten samples classifies as ema-2 genotype A, nine were also classified as 18S rRNA genotype A and one as genotype C. Of the two samples classified as ema-2 genotype C, one was 18S rRNA genotype C and the other genotype D (Table S1). The latter sequence was omitted from the phylogenetic analysis due to insufficient sequence length.

Classification of B. caballi rap-1 gene

Babesia caballi rap-1 gene was successfully amplified and sequenced from 19 horses, six showing clinical signs of disease and 13 subclinical carriers (MK346858-MK346873, MN635788). A long fragment of over 1400 bp was obtained from all clinical and three subclinical cases; and a shorter fragment, between 250 and 1000 bp from the remaining samples.

Of the two B. caballi rap-1 genotypes (A and B, with genotype A subdivided into subgroups A1 and A2) [12], four clinical and ten subclinical sequences obtained in this study were classified as genotype A1, while two clinical and three subclinical sequences were classified as genotype A2. All three subclinical isolates characterized as A2 originated from the same farm (Fig. 6).

All clinical samples were classified based on both their 18S rRNA and rap-1 genes and showed association between the two. All four samples that were characterized as 18S rRNA genotype B1 were also classified as rap-1 genotype A1, while the remaining two samples were 18S rRNA B2 and rap-1 A2 (Table S1).

Discussion

Diagnosis of EP as the cause of clinical disease can be challenging in endemic areas, where the percentage of serologically and molecularly positive horses is high, and the detection of parasites does not necessarily imply on the cause of non-specific clinical signs [32]. Therefore, in clinical cases suspected as EP, quantitative evaluation of parasite load, using molecular tools, may assist in determining a threshold for cause of disease decision. Here we demonstrate that clinically infected horses with either parasite of EP have significantly higher parasite loads and lower PCV than subclinically infected horses. This is intuitive, as merozoite replication in erythrocytes causes hemolysis, the main clinical manifestation of EP. Thus, higher parasite loads may induce increased hemolysis that will be reflected in lower PCV. Parasite loads of both clinical and subclinical horses were generally lower in cases

of B. caballi infection than in cases of T. equi infection. This may explain the milder clinical disease in B. caballi infections compared to T. equi infections, and to the possible natural clearance of B. caballi parasitemia without treatment, while T. equi carriage is usually life-long [1, 2].

To determination whether T. equi is the probable cause of disease in suspected clinical cases we established a clear cutoff ($P < 0.001$) between clinical (0.12–5.3% PE) and subclinical ($3*10^{-4}$-$5*10^{-3}$% PE) cases. The parasitemia values in our study concur with published subclinical range (1.99–1000 parasites per µl blood [33], equivalent to $2.2*10^{-5}$ to 0.011% PE). In clinical cases T. equi parasitemia ranges between 1–7% PE, and may reach up to 95% [1, 34], however, we had clinical cases with parasitemia as low as 0.12% PE, which also manifested in low PCV.

Three of six clinically B. caballi infected horses showed parasitemia below the documented range (0.1–10% PE [1, 34]). Parasitemia in subclinical carriers of B. caballi ranged between 0.0001 and 0.0012% PE, which was significantly lower than the clinical cases ($P < 0.001$). To the best of our knowledge, no previous study quantified B. caballi parasitemia in subclinical horses. Although in this group the difference between clinical and subclinical parasitemia was less distinct, it still manifested in lower PCV, and allowed to establish a cutoff value to identify B. caballi as the probable cause of clinical disease.

Despite the limited number of cases included in the quantitative analysis, the highly significant results may serve as first indication that qPCR may serve as a diagnostic tool. Additional data should be collected to validate this method for clinical use.

All seven clinical cases which originated from different farms and geographical locations were classified as T. equi 18S rRNA genotype A. The A genotype is not the most prevalent in our area (30% of subclinical horses in this study, 33% in a previous study), and is rarely found in highly endemic farms [10]. Thus, although the number of cases was limited, genotype A may be associated with clinical disease. Genotype A was previously isolated from horses in both endemic and non-endemic countries [5, 10, 31, 35], it was isolated in two outbreaks in the US [36], and was found to be associated with clinical and seropositive cases in Italy [19]. Interestingly, genotype A was the predominant genotype isolated from ticks collected from horses in Israel, including in farms in which this genotype was not isolated from horses (unpublished data). It is possible that this genotype is more adapted to the tick vector environment and encounter an active barrier at the horse stage, meaning that genotype A is more likely to lead to clinical disease, while genotypes B, C and D are more likely to result in subclinical infection [19]. With the recent concerns regarding the classification of Theileria species according to the 18S rRNA gene [6, 7], it is possible that this “A genotype” is the cause of the “classic” equine theileriosis, while other genotypes may represent closely related, less pathogenic, species or subspecies. More comprehensive genetic investigation of different genotypes is required to support this hypothesis.

In an attempt to partially address these issues, we classified T. equi according to three different genes: 18S rRNA, ema-1 and ema-2, as the last two loci had sufficient number of published sequences for comparative analysis. However, we could not amplify ema-1 and ema-2 from all samples, probably due to

polymorphism in the primer sites or the sensitivity of the PCR assay, and most of the successfully sequenced amplicons were from isolates of 18S rRNA genotype A, as previously reported [19]. The over represented 18S rRNA genotype A may be the result of the higher parasitemia in the clinically infected horses, enabling better detection in PCR. Nevertheless, using qPCR, ema-1 gene was detected in all samples, strengthening parasite identification.

The 18S rRNA classification in subclinical horses resulted in prevalence of genotypes D (57.5%), A (30%) and C (12.5%) as previously described in our area [10] strengthen the statistical power of the data with a larger sample size. Sequence analysis of both ema-1 and ema-2 did not reveal much polymorphism in these loci within a geographic area, as was previously demonstrated [10, 14, 16–18, 19]. Only 16 ema-2 sequences were available for classification, mostly from India and the US along with four sequences from Nigeria (generated by our group, Acc. No. MN519202-MN519205). Although this gene had low variability, we identified three distinct genotypes, which also differ in their amino acid sequences. This variability may be important if it affects immune response and may lower the sensitivity of ema-2 based ELISA assays [18].

Genetic classification of B. caballi is limited, with two 18S rRNA genotypes identified in South Africa [5]. We were unable to amplify this gene from the subclinical horses, since all were co-infected with T. equi, and the primers are not species specific. Therefore, we used the rap-1 gene which is specific to B. caballi and is fairly conserved, with some heterogenicity between American and Asian-African strains [12–14, 19]. Two 18S rRNA sub-genotypes were identified in clinical cases, which correlated with the rap-1 sub-genotypes of the same samples. Comparison of the rap-1 gene between clinical and subclinical cases did not reveal differences in parasite genotypes in relation to clinical disease.

Conclusion

This study provides in-depth molecular comparison between parasites of clinical and subclinical cases of EP. Quantitative molecular tools which assess parasite loads may help clinicians decide whether EP is the cause of the presenting clinical signs, since significantly higher parasitemia was associated with clinical disease. This tool, however, should be standardized for different laboratories and geographical areas.

T. equi genotype A (based on 18S rRNA classification) is associated with clinical disease, while no such association was found for B. caballi. Future studies on parasites classification should be established based on multi locus sequence typing (MLST), in order to distinguish between closely related organisms or genotypes which may differ in their pathogenicity.

Abbreviations

B – Babesia

ema - equi merozoite antigen

EP – equine piroplasmosis

gcn – gene copy number

PCV – packed cell volume

PE – parasitized erythrocytes

T – Theileria

TS - total solids

rap - rhoptry associated protein

Additional Files

Table S1

Detailed description of the study population. The characteristics of the horses and associated parasites of *B. caballi* and *T. equi* clinically (Y) and subclinically (N) infected horses. The sex, breed, age, geographical area and packed cell volume (PCV) are specified for each horse, along the molecular classification of isolated parasites according to the 18S rRNA, *rap-1* (*B. caballi*), *ema-1* and *ema-2* (*T. equi*(. S-stallion, M-mare, G-gelding; Ar-Arabian, TWH–Tennessee Walking Horse, QH-Quarter Horse, WB-Warmblood, An-Andalusian; GH-Golan Heights.

Declarations

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Ethics approval

All samples were obtained with owner's permission and the study was approved by the Research Committee of the Koret School of Veterinary Medicine – Veterinary Teaching Hospital (KSVM-VTH/23_2014).

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional file.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YG and AS conceived, designed, and supervised the study. STL designed the study, conducted the field work, analyzed the data, and drafted the manuscript. STL, HL, YK and MS preformed the lab experiments. All authors read and approved the final manuscript.

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Tables

Table 1

The PCR primers and probes used in this study.

Primer	Sequence 5'-3'	Target gene	Amplicon size (bp)	Reference
Bec-UF2	TCGAAGACGATCAGATACCGTCG	*T. equi/ B. caballi* 18S rRNA	400	20
Equi-R	TGCCTTAAACTTCCTTGCGAT			
Bc9_RAP2F	ACTAGCGACCCCAACGCTACTGAC	*B. caballi Rap-1*	400	11
Bc9_RAP2R	TTGGAGCATGAAGTCCTTCAGC			
RT_EMAF	CCGGCAAGAAGCACAYCTT	*T. equi ema-1*	59	14
RT_EMAR	TGCCATCGACGAYCTTGAG			
RT_EMA probe	6-FAM-TCCAGGCAAGCGC-MGB			
Bc_18SF402	GTAATTGGAATGATGGCGACTTAA	*B. caballi* 18S rRNA	95	22
Bc_18SR496	CGCTATTGGAGCTGGAATTACC			
Bc_18SP	6-FAM-CCTCGCCAGAGTAA-MGB			
NBabesia1F	AAGCCATGCATGTCTAAGTATAAGCTTTT	*T. equi* 18S rRNA	1600	25
18SRev-TB	GAATAATTCACCGGATCACTCG			24
BT18S2F	GGGTTCGATTCCGGAGAGGG	*T. equi* 18S rRNA	800	25
BT18S2R	CCCGTGTTGAGTCAAATTAAGCCG			24
BT18S3F	GGGCATTCGTATTTAACTGTCAGAGG	*T. equi* 18S rRNA	800	25
BT18S3R	CCTCTGACAGTTAAATACGAATGCCC			5
EMA-1F	GCATCCATTGCCATTTCGAG	*T. equi ema-1*	750	20
EMA-1R	TGCGCCATAGACGGAGAAGC			
EMA-2F	AATGTTGAGCAAGTCCTTCG	*T. equi ema-2*	800	Kumar et al., 2018
EMA-2R	TTAGTAGAACAAAGCAACGGC			
Bc9_RAPF	AGCAGTGCTGTATATGTCTGTGTC	*B. caballi rap-1*	1500	11
Bc9_RAPR	GCTGATGCGATGTGTGTCGTAGG			

Table 2

Characteristics of the horses participated in this study. The number (N) of clinical and subclinical isolates analyzed in this study, sex and breed distribution, mean age and mean packed cell volume (PCV) are specified for each group. Ar-Arabian, TWH–Tennessee Walking Horse, QH-Quarter Horse, WB-Warmblood, An-Andalusian.

	B. caballi		*T. equi*	
	Clinical	**Subclinical**	**Clinical**	**Subclinical**
N	6	13	7	40
Mare	3	2	5	16
Gelding	3	10	2	23
Stallion		1		1
Ar			1	
TWH	1		1	
QH	3		5	4
WB	1			
An		1		
Pony				2
Mixed	1	12		34
Mean age	6.20	6.90	10.57	9.17
Mean PCV	19.75	30.70	19.42	33.82

Table 3

Estimates of the evolutionary divergence within and between *T. equi ema-2* genotypes. The average number of base substitutions per site is shown. A total of 782 nucleotide positions of 29 nucleotide sequences were analyzed. The analyses were conducted using Kimura-2 parameter+G medel in MEGA7.

	Within group	Between groups	
Genotype		**A**	**B**
A	0.000		
B	0.004	0.011	
C	0.001	0.059	0.055

Figures

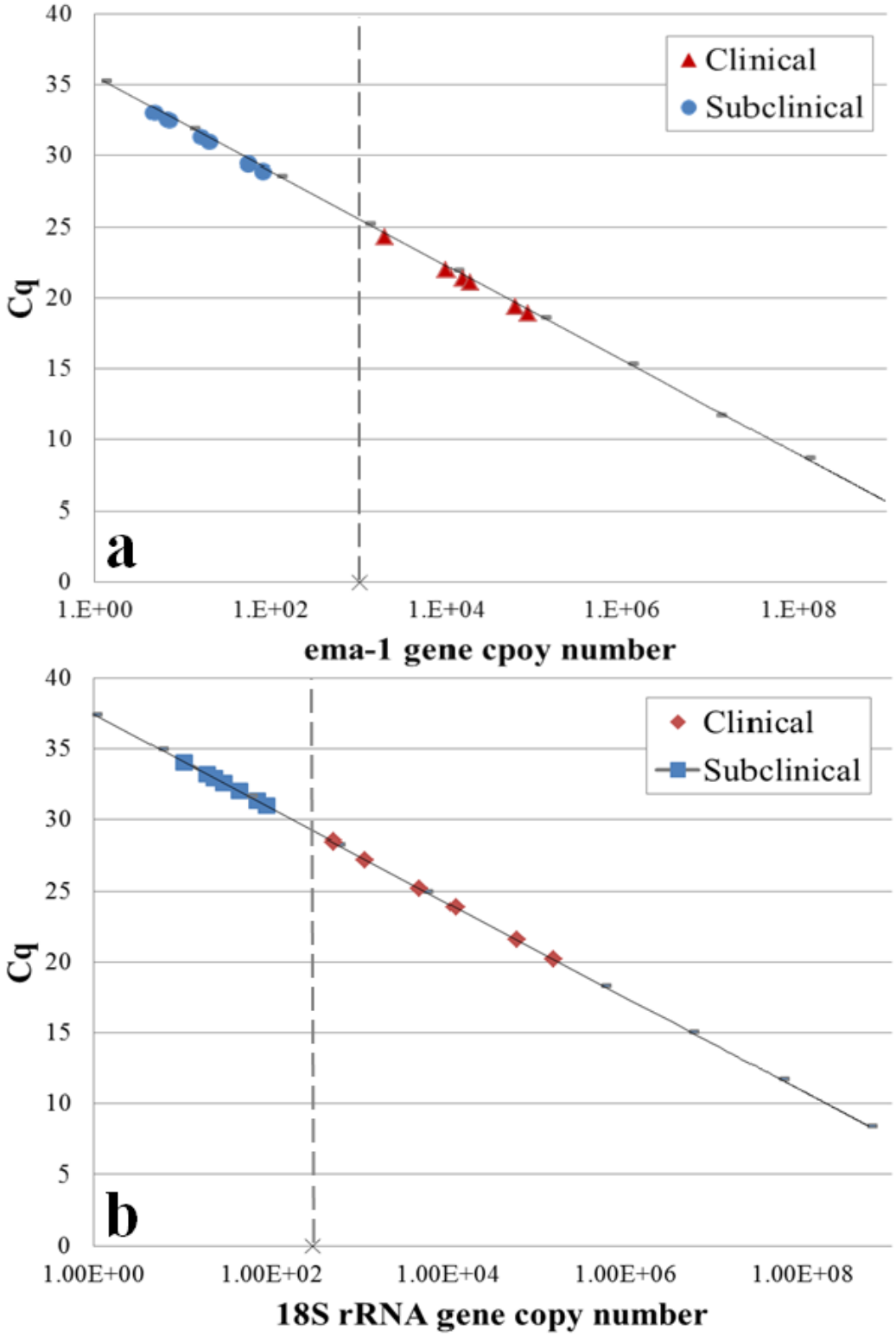

Figure 1

Theileria equi (A) and B. caballi (B) parasite loads in clinical (red) and subclinical (blue) horses, as determined by qPCR. For each parasite a standard curve (black line) was created using serial dilutions of a clean PCR product of each gene (gray marks). Parasite gene copy number from field samples was calculated from the quantification cycle (Cq) and the standard curve. A diagnostic cutoff distinguishing

between clinical and subclinical cases of each parasite was determined by ROC analysis, and the cutoff value is marked by a vertical dashed line.

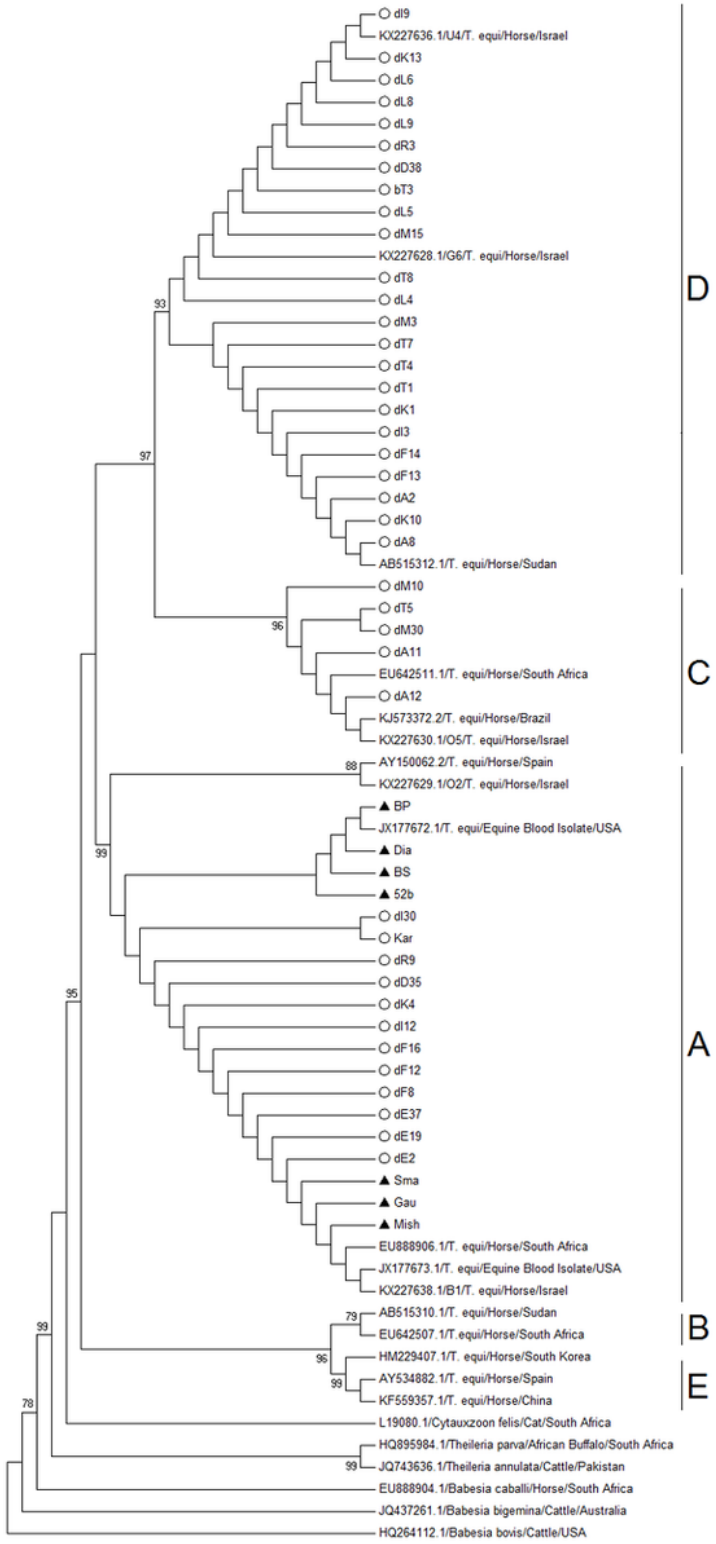

Figure 2

Phylogenetic analysis of 1079 nucleotide positions of T. equi 18S rRNA gene sequences obtained from seven clinically infected horses (▲) and forty subclinically infected horses (●) (sample names as detailed in Table S1) along with twenty three additional published sequences (GenBank

AC#/parasite/host/location). The phylogenetic tree was constructed by maximum likelihood method based on the Tamura-Nei model with gamma distribution (+G) and 1000 bootstrap replicates. The percentage of trees in which the associated samples clustered together is shown next to the branches when it was above 70%. The analysis was constructed in MEGA7.

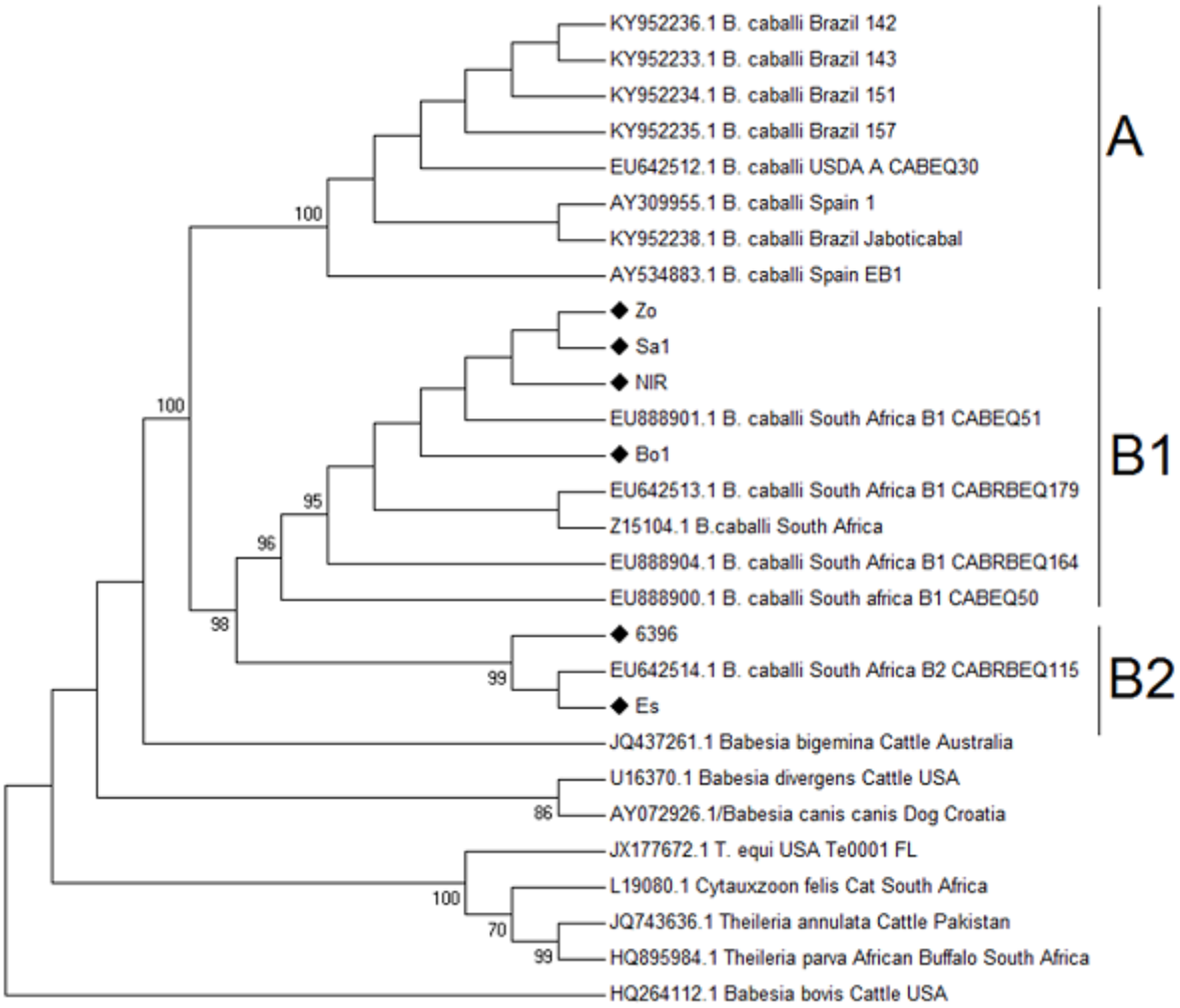

Figure 3

Phylogenetic analysis of 1212 nucleotide positions of B. caballi 18S rRNA gene sequences obtained from six clinically infected horses (♦) (sample names as detailed in Table S1), along with 22 additional published sequences (GenBank AC#/parasite/host/location). The phylogenetic tree was constructed by maximum likelihood method based on the Tamura-Nei model with gamma distribution (+G) and invariable sites (+I), and with 1000 bootstrap replicates. The percentage of trees in which the associated samples clustered together is shown next to the branches when it was above 70%. The analysis was constructed in MEGA7.

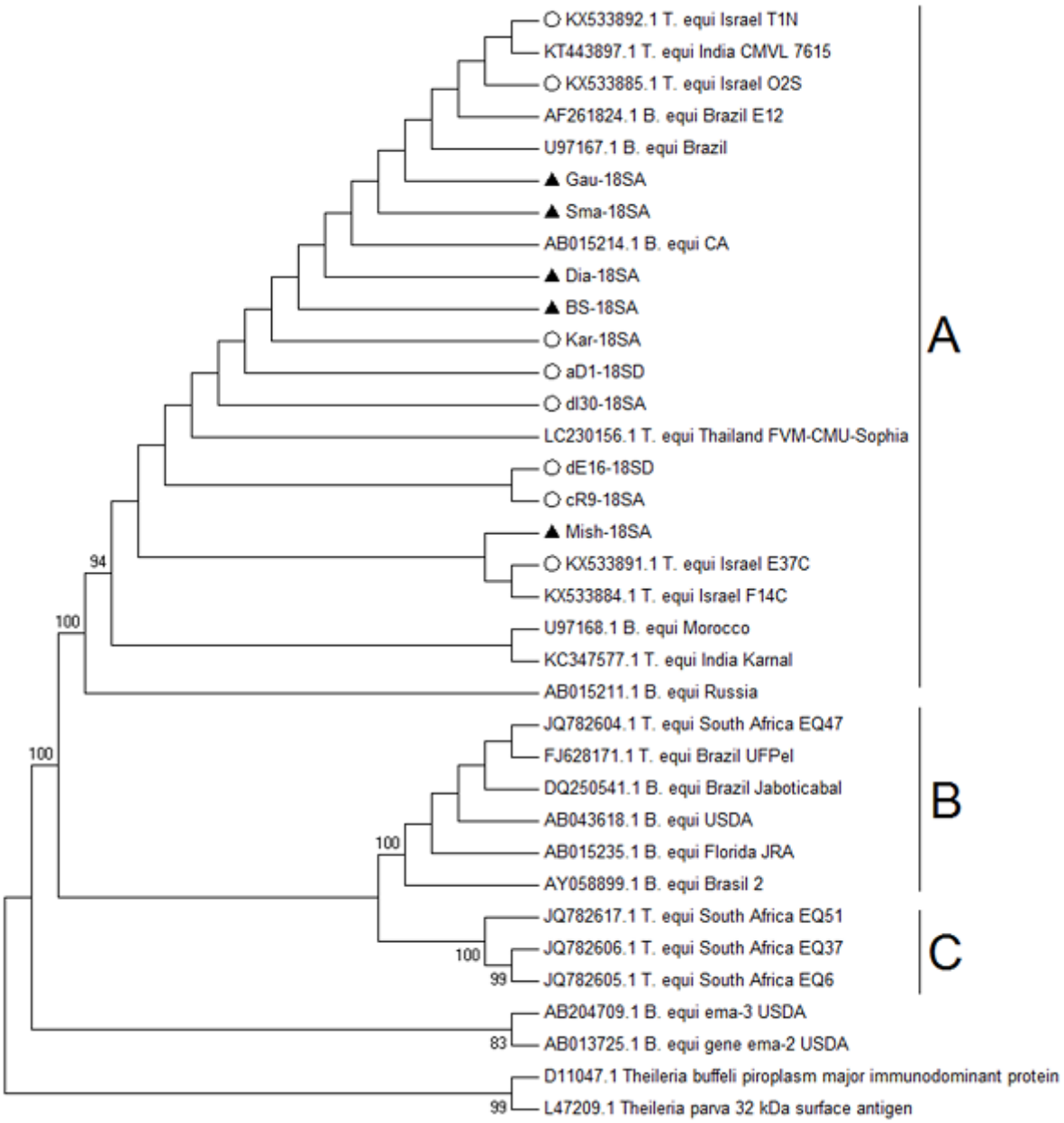

Figure 4

Phylogenetic analysis of 400 nucleotide positions of T. equi ema-1 gene sequences obtained from five clinically infected horses (▲) and five subclinically infected horses (●) (sample names as detailed in Table S1), along with 46 additional published sequences (GenBank AC#/parasite/host/location). The classification of each sample according to its 18S rRNA gene is states near the sample name (-18SX). The phylogenetic tree was constructed by maximum likelihood method based on the Kimura 2-parameter model with consideration on invariable sites (+I) and 1000 bootstrap replicates. The percentage of trees in which the associated samples clustered together is shown next to the branches when it was above 70%. The analysis was constructed in MEGA7.

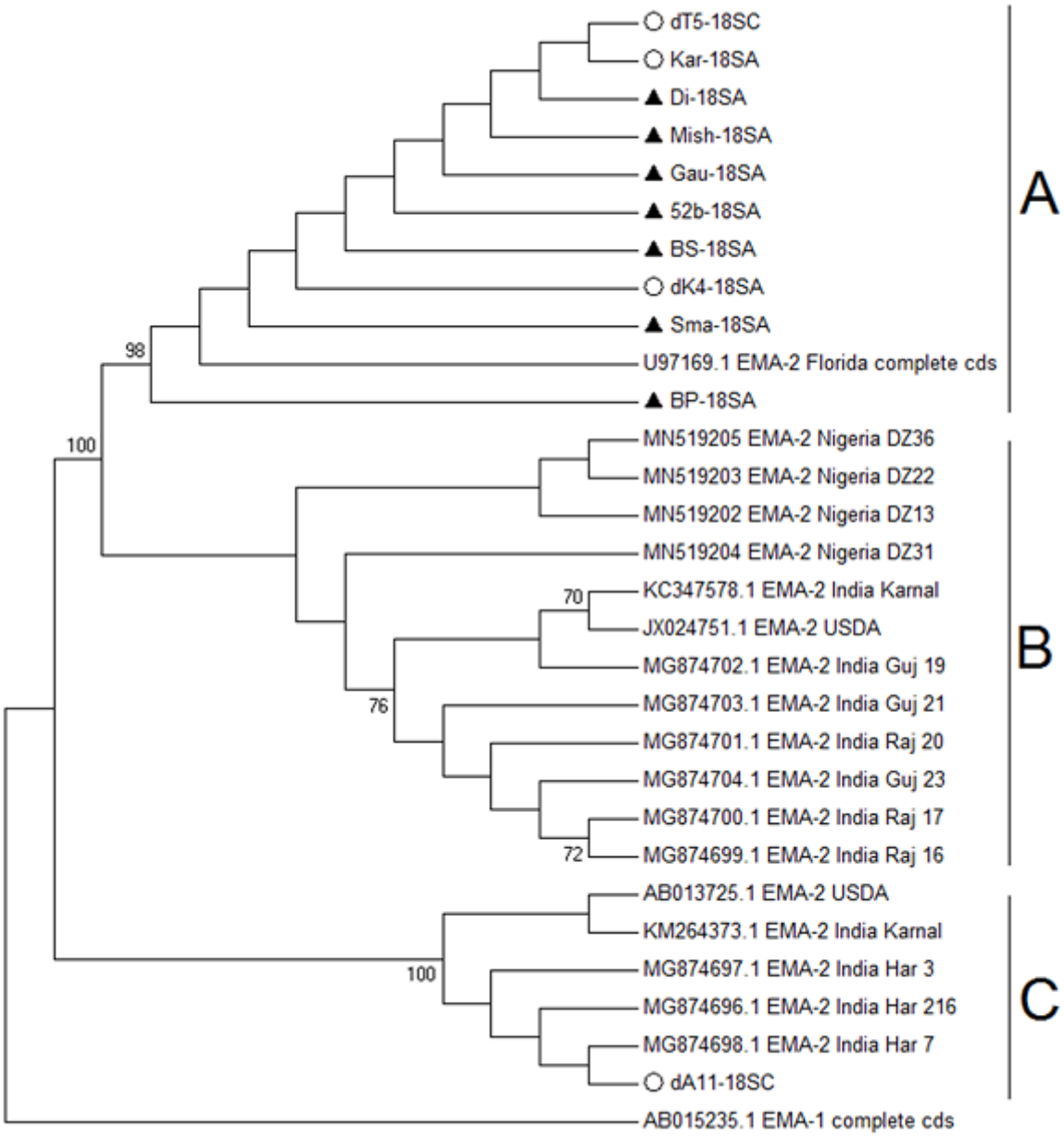

Figure 5

Phylogenetic analysis of 782 nucleotide positions of T. equi ema-2 gene sequences obtained from seven clinically infected horses (▲) and four subclinically infected horses (●) (sample names as detailed in Table S1), along with 19 additional published sequences (GenBank AC#/parasite/host/location). The classification of each sample according to its 18S rRNA gene is states near the sample name (-18SX). The phylogenetic tree was constructed by maximum likelihood method based on the Kimura 2-parameter model with consideration on invariable sites (+I) and 1000 bootstrap replicates. The percentage of trees in which the associated samples clustered together is shown next to the branches when it was above 70%. The analysis was constructed in MEGA7.

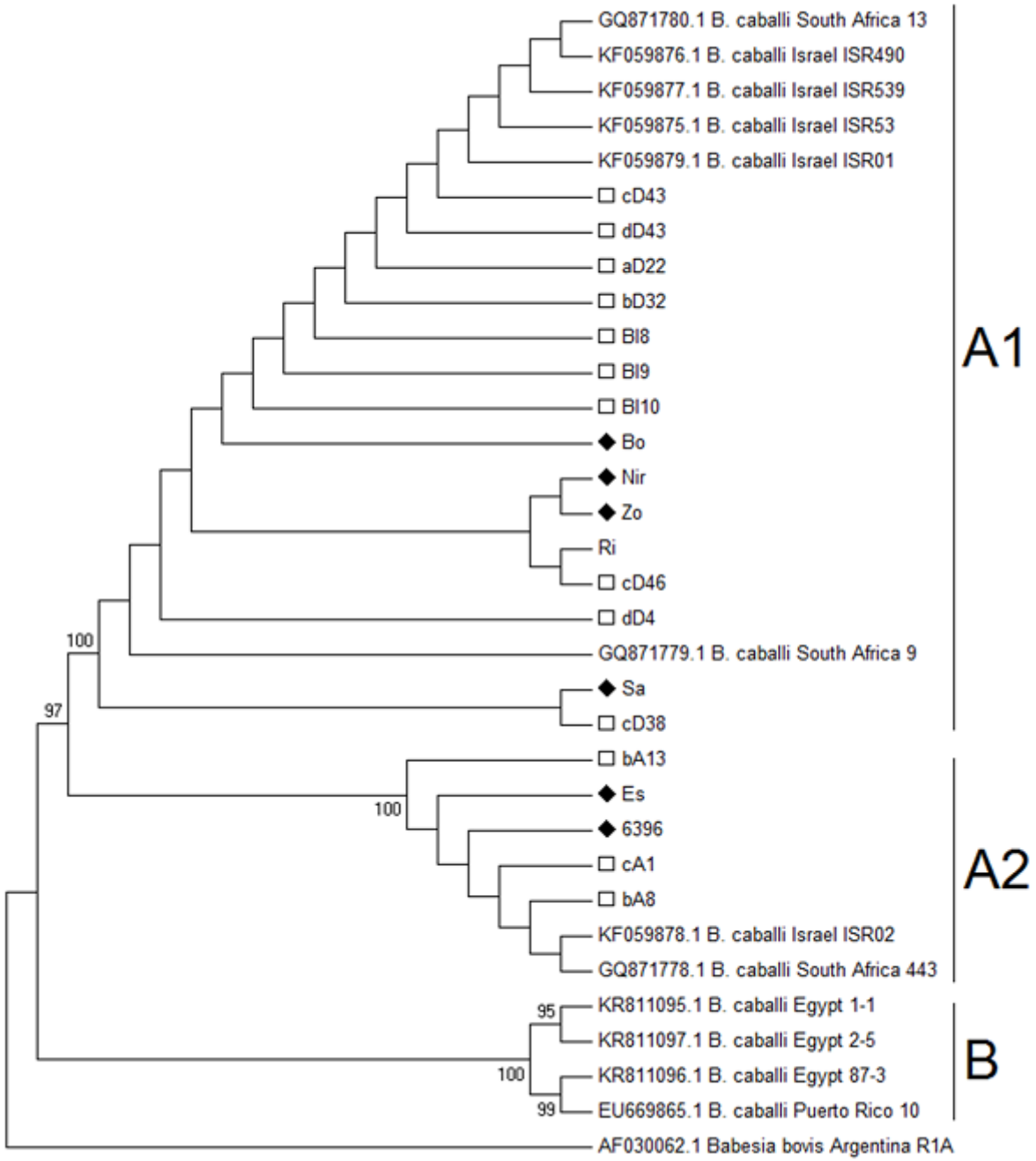

Figure 6

Phylogenetic analysis of 251 nucleotide positions of B. caballi rap-1 gene sequences obtained from six clinically infected horses (♦) and 13 subclinically infected horses (□) along with 14 additional sequences from the GenBank. The phylogenetic tree was constructed by maximum likelihood method based on the Kimura 2-parameter model with consideration on invariable sites (+I) and 1000 bootstrap replicates. The percentage of trees in which the associated samples clustered together is shown next to the branches when it was above 70%. The analysis was constructed in MEGA7.

Supplementary Files

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- graphicalabstract4.png