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Original article

A multinested PCR for detection of the equine piroplasmids *Babesia caballi* and *Theileria equi*

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A B S T R A C T

Two haemoparasites, *Theileria equi* and *Babesia caballi*, cause equine piroplasmosis (EP), one of the most prevalent tick-borne diseases in horses. The main aim of the present study was to develop and evaluate a multinested PCR (mn-PCR) for simultaneous detection of the equine piroplasmids *T. equi* and *B. caballi*, by amplification of five genetic markers (*18S rRNA*, *β-tubulin*, *cytB*, *EMA-1* and *RAP-1*). This novel assay detected a high prevalence of equine piroplasmids in 235 horse blood samples collected in Castilla-León and Extremadura, Spain. The overall prevalence of infection with equine piroplasmids by mn-PCR was 72.8% (171/235), with 66.0% (155/235) of the animals positive for *T. equi* and 29.4% (69/235) positive for *B. caballi*. The seroprevalence obtained by cELISA for the same set of samples was lower than the infection prevalence recorded by mn-PCR, for either of the two equine piroplasmids (62.6%) as well as for *T. equi* alone (61.7%) or *B. caballi* alone (3.8%). There was high agreement among the mn-PCR and cELISA assays for diagnosis of EP caused by *T. equi* (κ = 0.83) but not for *B. caballi* (κ = 0.06). A phylogenetic analysis based on the *RAP-1* gene of *B. caballi* showed that the strains from Spain clustered with those from Israel.

1. Introduction

Equine piroplasmosis (EP) is a tick-borne disease characterized by fever, jaundice, depression and anorexia. This illness is caused by two haemoprotozoan parasites, *Babesia caballi* (Nuttall and Strickland, 1910) and *Theileria equi* (Mehlhorn and Schein, 1998). EP is widespread in tropical and subtropical regions (Uilenberg, 2006). Detection of chronically infected animals is important to avoid further spread of the disease through international horse trading (Ayala-Valdovinos et al., 2014). Thus, sensitive and specific techniques for detecting piroplasmid infections are needed.

Indirect methods are usually used to diagnose carrier horses. The most common indirect methods are indirect fluorescent antibody tests (IFAT) and competitive enzyme-linked immunosorbent assays (cELISA). The cELISA is based on recombinant proteins from the *EMA-1* gene of *T. equi* and the *RAP-1* gene of *B. caballi* (Knowles et al., 1992; Brüning et al., 1997; Kappmeyer et al., 1999; Xuan et al., 2001, 2002). The cELISA for detecting *T. equi* was developed using a recombinant *EMA-1* protein and specific monoclonal antibodies (Knowles et al., 1991, 1992). Meanwhile, the cELISA for *B. caballi* uses specific monoclonal antibodies and a recombinant *RAP-1* protein (Kappmeyer et al., 1999). Monoclonal antibodies and recombinant proteins increase sensitivity and specificity, but serological procedures have disadvantages due to cross reaction with other piroplasmids (Friedhoff and Soulé, 1996). Application of molecular along with serological techniques could detect active and passive infections (Munkhjargal et al., 2013; Sgorbini et al., 2015), and may increase sensitivity and specificity (Ferreira et al., 2016). Often, the combination chosen is Polymerase Chain Reaction (PCR) and cELISA. PCR assays are useful tools for diagnosis of EP (Posnett and Ambrosio, 1991; Posnett et al., 1991). Several genes have been targeted by PCR to detect equine piroplasmids, including the *18S rRNA* (Bashiruddin et al., 1999; Rampersad et al., 2003; Alhassan et al., 2005; Kim et al., 2008) and the *β -tubulin* (Cacciò et al., 2000; Montes et al., 2017a) genes for *T. equi* and *B. caballi*. The *EMA-1* gene (Nicolaiewsky et al., 2001; Ueti et al., 2003) was used exclusively for *T. equi* and the *RAP-1* (rhoptry associated protein) gene (Bhoora et al., 2010b) exclusively for *B. caballi*.

Several PCR-based methods have been standardized for EP diagnosis (Alhassan et al., 2005; Nicolaiewsky et al., 2001; Rampersad et al., 2003; Kim et al., 2008; Baptista et al., 2013; Kizilarslan et al., 2015; Mans et al., 2015); they were shown to be more sensitive, specific and useful than other techniques such as the fixation test (CFT) or the IFAT for the diagnosis of equine apicomplexan protozoa (Böse et al., 1995). The main goal of the present research was to develop a robust

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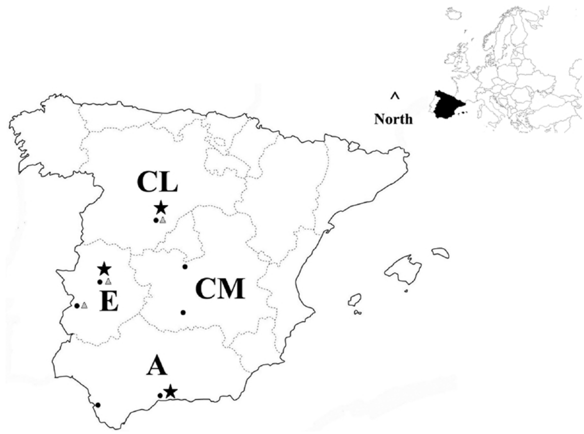

Fig. 1. Map of Spain showing locations where the samples were collected (Regions: A – Andalucía; CL – Castilla-León; CM – Castilla-La Mancha; E – Extremadura. Black dots = samples for the development of the mn-PCR; Grey triangles = samples from asymptomatic horses; Black stars = samples for DNA sequencing of the *RAP-1* gene).

multinested PCR protocol for simultaneous detection of *T. equi* and *B. caballi* infections, and compare it with the commonly used commercial cELISA kit. In addition, the *RAP-1* gene from *B. caballi* was characterized within several Spanish isolates.

2. Materials and methods

2.1. Blood sample collection

Twenty-four blood samples (21 from infected horses during the acute phase and 3 from negative animals) were used to set up the mn-PCR. To note, the horses in the acute phase presented clinical symptoms and were diagnosed by microscopic observation of stained blood smears (15 animals were *T. equi*-positive; 5 blood samples were *B. caballi* positive and 1 sample had mixed infection). In addition, the 24 samples were tested by IFAT and cELISA to confirm the results. These samples were collected in several regions of Spain: Castilla-León (n = 5), Castilla-La Mancha (n = 3), Extremadura (n = 9) and Andalusia (n = 7).

For the following comparative survey (Fig. 1), 235 blood samples were collected from asymptomatic horses from three different herds; two from Extremadura (n = 30 horses) and the third one from Castilla-León (n = 205 horses). Blood samples obtained by jugular venipuncture were collected into blood-collection tubes (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ, USA). Two samples were drawn from each horse: one tube, with EDTA, for blood to be used for molecular assays and the other one, without anticoagulant, for serology. All samples were kept refrigerated for transportation to our laboratory. Serum samples were obtained from clotted blood samples by centrifugation and then frozen (-20 °C) until use. Blood samples were stored at -70 °C until DNA extraction. Samples were collected from March to June 2014. The owners of the animals gave their permission to obtain the blood samples and the necessary operations were carried out by assistant veterinarians, following the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. DNA extraction

DNA was extracted from approximately 250 µL of red blood cells from the blood samples. The extraction was carried out as described previously (Millar et al., 1988; Fernández-García, 2012) using Zymo-Spin IIC columns® (Zymo Research Corporation, Irvine, CA, USA) to purify the nucleic acids, which have a binding capacity limit of 25 µg DNA. The DNA samples were diluted in 150 µl of DNase/RNase-free water.

2.3. Development of the multinested PCR (mn-PCR)

The mn-PCR technique developed here was based on seven gene targets (four gene targets for *T. equi* and three for *B. caballi*). Oligonucleotide primers were designed using the alignment of available sequences in GenBank from the *18S rRNA*, the *β-tubulin*, the *EMA-1* and

Table 1
First mn-PCR primers (outer primers) and second mn-PCR primers (inner primers) according to target species and genes. (R = reverse and F = forward). Amplicon sizes are shown in bp.

Target species	First mn-PCR primers outer				
	Gene	Primer designation	Sequence 5´-3´	specie (bp size)	Reference
B. caballi and *T. equi*	18S *rRNA*	BecUR	CGGTATCTGATCGTCTTCGA	*B. caballi* (904)	Alhassan et al. (2005)
		BecUF1	TGATCCTGCCAGTAGTCAT	*T. equi* (956)	Alhassan et al. (2005) Modified in this research
B. caballi and *T. equi*	*β-tubulin*	β-Tub R2	CAGCTTTAGRGTTCKGAAGCARAT	*B. caballi* (828)	Montes et al. (2017a)
		β-Tub-outerF2	GAATGAGGGARATCGTWCACA	*T. equi* (718)	Present paper
T. equi	*EMA-1*	EMA1-outerR	TGTCGTCACTTAGTAAAATAGAG	*T. equi* (828)	Present paper
		EMA1-outerF	ATGATTTCCAAATCCTTTGC		Present paper
T. equi	*cytB*	cytB-outerR	ATTTACCTTGGTCTTGGTATT	*T. equi* (213)	Present paper
		cytB-outerF	AATGTAGTATATAAAAYCTTTGC		Present paper
B. caballi	*RAP-1*	RAP1-outerR	GCTTCATGTACCACTTCTTATAC	*B. caballi* (891)	Present paper
		RAP1-outerF	GCGCCCTCTTGCTYGTAG		Present paper
	Second mn-PCR primers inner				
T. equi	18S *rRNA*	18Sequi-innerR	AAAGTATTCAAGGCAAAAGC	*T. equi* (143)	Present paper
		18Sequi-innerF	TCGTAGTTGAATTTCTGCTG		Present paper
T. equi	*β-tubulin*	GME-Tub-innerR	GGTACCTGGTTCCAAATCC	*T. equi* (185)	Present paper
		GME-Tub-innerF	ATCCGGTAAGTTTGTGTCTAC		Present paper
T. equi	*EMA-1*	EMA1-innerR	TGTCCTTGATGTGCCTGAC	*T. equi* (274)	Present paper
		EMA1-outerF	ATGATTTCCAAATCCTTTGC		Present paper
T. equi	*cytB*	cytB-innerR	CTTGGTCTTGGTATTCTGG	*T. equi* (207)	Present paper
		cytB-outerF	AATGTAGTATATAAAAYCTTTGC		Present paper
B. caballi	18S *rRNA*	18Scab-innerR	TGCTGAAGTATTCAAGACAAA	*B. caballi* (607)	Present paper
		18S-cab-innerF	AGTTTCTTTGGTATTCGTTTTC		Present paper
B. caballi	*β-tubulin*	GMB-TubR	AGTTGTCRGGYCTGAAGAGT	*B. caballi* (346)	Montes et al. (2017a)
		GMB-Tub-innerF	ACCCGGTAAGTCGTTAAACC		Present paper
B. caballi	*RAP-1*	RAP-1outerR	GCTTCATGTACCACTTCTTATAC	*B. caballi* (566)	Present paper
		RAP1-innerF	GTACCAACCGCTGACCCTTC		Present paper

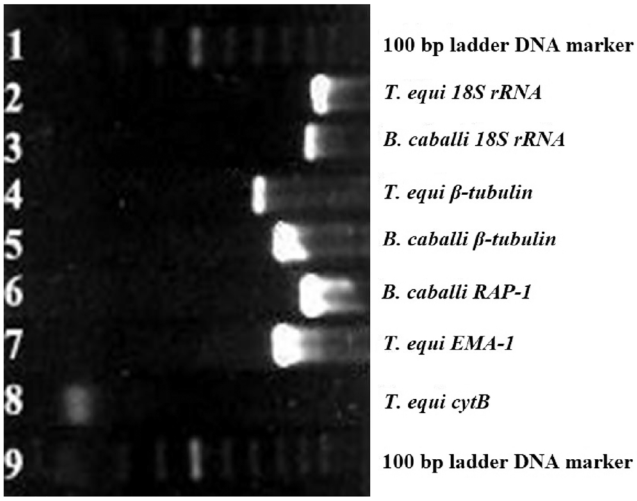

Fig. 2. First mn-PCR amplicons. Lane 1 to 9: 100 bp ladder DNA marker, *T. equi 18S rRNA* gene, *B. caballi 18S rRNA* gene, *T. equi β-tubulin* gene, *B. caballi β-tubulin* gene, *RAP-1* gene, *EMA-1* gene, *cytB* gene and 100 bp ladder DNA marker.

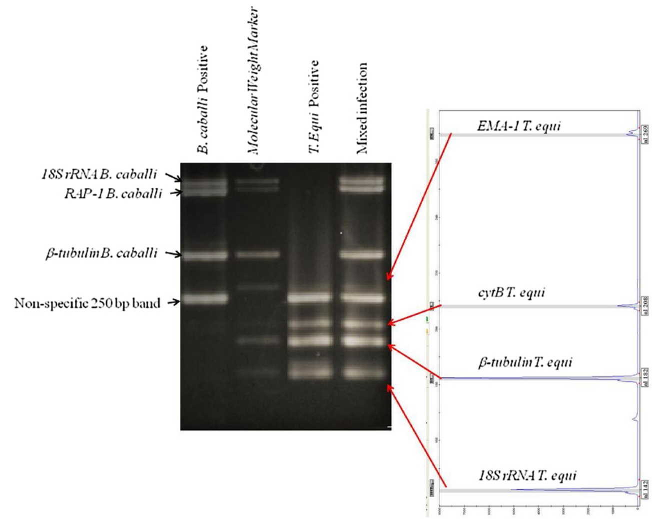

Fig. 3. Simultaneous detection of *B. caballi* and *T. equi* from equine blood DNA by mn-PCR. Left: Conventional agarose gel. Right: Capillary electrophoresis using FAM-labelled F-inner primer for each gene of *T. equi*.

the *cytB* (mitochondrial Cytochrome *B*) genes of *T. equi* and for the *18S rRNA*, the *β-tubulin* and the *RAP-1* genes (accession number in Table S1) for *B. caballi*. The downloaded sequences were aligned using MEGA 6.05 software (Tamura et al., 2013). The primary mn-PCR was performed with the named outer primers and the secondary mn-PCR with the named inner primers, while *EMA-1*, *cytB* and *RAP-1* inner pairs worked as seminested PCR (Table 1). A negative control consisting of the reaction mix and DNase/RNase-free water and two positive controls (for *T. equi* and *B. caballi*) were included for each batch of samples.

The development of the mn-PCR assays was performed in several steps using samples obtained from horses with known acute infections (see section 2.1). In the first step all the outer primer pairs were used as individual pairs with *B. caballi* and *T. equi* DNA obtained from the acutely infected horses (Fig. 2). The amplifications were successful except cytB-outer when it was used with the *B. caballi* DNA. Therefore, this last outer primer pair was unsuccessful for the mn-PCR regarding this parasite. In the second step, all inner primer pairs were used as individual pairs using their corresponding parasite DNA template (Table 1).

Different amplicon sizes were conceived to yield a bar-coding pattern after secondary mn-PCR when run in an agarose gel. Thus, the *B. caballi* amplicons were above 300 bp whereas the *T. equi* bands occurred below 300 bp (Fig. 3). In a mixed infection, a complete bar-coding should be expected. The amplicon sizes of the first mn-PCR and the second mn-PCR are shown in Table 1.

An unspecified band of around 250 bp was observed after the second mn-PCR below the *T. equi EMA-1* fragment; for this reason, a specific molecular size marker was built using amplicons from conventional PCR using inner primer pairs (seven single PCR) and the DNA template from acute infected horse for *B. caballi* and *T. equi*. Thus, a precise and specific molecular size marker was obtained (Fig. 3).

The first mn-PCR was performed as follows: 32.5 μl of a mixture containing 1x PCR buffer, 2.5 μl of each primer pair for the *18S rRNA* gene (5 μM), the *cytB* gene (5 μM), the *RAP-1* gene (5 μM), the *β-tubulin* gene (6 μM), the *EMA-1* gene primers (6 μM), 2.5 μl of 2 mM dNTP mixture, 3.25 mM $MgCl_2$, 0.5 unit of BioTaq™ DNA polymerase (Ecogen, Barcelona, Spain) and 7.5 μl of DNA template. This mixture was heated for an initial denaturation of 5 min at 94 °C; then 37 cycles of 45 s at 94 °C, 1 min at 52 °C and 1 min at 72 °C; with a final extension of 7 min at 72 °C. The first mn-PCR products were diluted 1:4 (2 μl in 8 μl of DNase/RNase-free water) and 1 μl of this dilution was added to 15 μl of each of two different second mn-PCR mixtures, that is, one for each parasite (*T. equi* or *B. caballi*). The *T. equi* mixture (mn-PCR A) contained 1x PCR buffer, 1.5 μl of 2 mM dNTPs, 2 mM $MgCl_2$ and 1.5 μl of each primer pairs 18Sequi-inner (12 μM), GME-Tub-inner (5 μM), EMA1-inner (9 μM) and cytB-inner primers (9 μM) added with 0.3 unit of BioTaq™ DNA polymerase (Ecogen). The *B. caballi* mixture (mn-PCR B) contained 1x PCR buffer, 1.5 μl of 2 mM dNTPs, 2 mM $MgCl_2$ and 1.5 μl of each primer pair 18Scab-inner (7 μM), GMB-Tub inner (4 μM) and RAP1-inner primers (5 μM) added with 0.3 unit of BioTaq™ DNA polymerase (Ecogen).The concentration of the primer pairs within each mixture was adjusted by thermal cycling mn-PCR reactions not only with pure DNA from *T. equi* and *B caballi* but also pooling both DNAs. Both mn-PCR (A and B) mixtures were heated to 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min and finally a post-elongation of 7 min at 72 °C. Then, each sample was amplified with each mixture and 7 μL of PCR products were pooled (A + B) for running them in the same lane in agarose gels (1.5%; 45 min at 100 v). The gels were stained with ethidium bromide for visibility (Fig. 3). All samples were amplified twice.

2.4. Sequencing and nucleotide evolutionary connection of the *RAP-1* gene

DNA sequencing of the *RAP-1* gene was carried out using three samples from acutely infected horses collected from different geographical locations: Málaga (Andalucía), Segovia (Castilla-León) and Cáceres (Extremadura) (Fig. 1). The PCR products were purified with ExoSAP-IT™ (GE Healthcare Limited, Little Chalfont, Buckinghamshire, UK). Sequencing was performed with the BigDye Terminator v.3.1 Cycle Sequencing Kit in both directions using RAP1-outerR and RAP1-outerF. *RAP-1* sequences of 769 bp were obtained using a 3130 ABI Genetic Analyzer, edited with the Applied Biosystems™ DNA Sequencing Analysis Software ver. 5.2 (Thermo Fisher, Waltham, MA, USA) and then aligned with sequence KF059879 (see Table S1) in MEGA 6.05 software (Tamura et al., 2013).

The phylogenetic relationships among the *B. caballi* sequences from the present study (accession numbers: MH538120 to MH538122) and 34 GenBank sequences (Table S2) were performed after truncating the alignment to the overlapping portion between these sequences. Thus, the overlapping portion expanded from nucleotides 290 to 647 (358 bp in length) in the complete *RAP-1* sequence (accession number AF092736).

A median-joining network was constructed with all these sequences to include the most parsimonious trees supported by the data (Bandelt et al., 1999; Forster et al., 2001; Polzin and Daneschmand, 2003), thus providing a genealogical inference that is more realistic and accurate than those obtained by strictly dichotomous trees (Posada and Crandall, 2001). The "median-joining (MJ) network" (Bandelt et al., 1999) created with the NETWORK 4.1.1.1 software (www.fluxus-technology.com) used the "star contraction" algorithm with a mutational distance radius (δ = 5) that collapsed haplotypes into haplogroups, displaying the inner structure of star-like clusters (Forster et al., 2001).

2.5. Inmunoenzymatic assays (cELISA)

A commercial cELISA test (VMRD®, Inc, Pullman, WA, USA) was employed to detect antibodies to *T. equi* and *B. caballi* in accordance with the recommendations made by the World Organization for Animal Health (OIE, 2017). Tests were carried out following the manufacturer´s instructions. Samples associated with percent inhibition (PI) values < 40% were considered negative, with PI values ≥40% being classified as positive. The optical density values were obtained using an automatic plate reader (Thermo Scientific Multiskan Ascent Microplate Reader®, Thermo Fisher, Waltham, MA, USA).

2.6. Inmunofluorescence antibody test (IFAT)

T. equi and *B. caballi* antibodies were also detected by IFAT. The antigen was obtained from the blood of naturally infected horses (parasitaemia > 3%). The preparation of the *T. equi* and the *B. caballi* antigen and the protocol test were conducted as previously described by Camacho et al. (2005). Slides were examined under a fluorescence microscope (Leica DMLS Binocular Microscope®, Leica Microsystems, Wetzlat, Germany) at 400x magnification. Positive and negative sera were included as controls in each batch of samples.

2.7. Statistical analysis

The diagnostic performance of the cELISA and mn-PCR tests for detection of equine piroplasmids was compared as follows. Fisher's exact test was used to determine whether the proportions for each of the four assays used to diagnose *B. caballi* and *T. equi* (cELISA-cab, cELISA-equi, mnPCR-cab and mnPCR-equi) showed significant differences with regards to horse age and gender (Table 2a). The diagnostic performances of the two methods used in the study (cELISA and mn-PCR), considering simultaneously both parasites and each parasite separately, were compared by the McNemar Chi-Square test for two related data sets as a whole as well as separately with regards to horse age and gender. The Odds Ratio (95% Confidence interval) was calculated using binary Logistic-Regression in IBM SPSS Statistics 15.00 for Windows (IBM Corporations, Armonk, NY, USA). A statistical test was considered significant at $p < 0.05$. The concordance between the serological and the mn-PCR methods for detection of *T. equi* and *B. caballi*, separately, was assessed using the Kappa´s coefficient (κ). All statistical analysis related to risk factors and the agreement between the diagnostic methods was performed with SPSS 15.0 software.

3. Results

3.1. Molecular analysis

Several PCR assays were carried out in order to set-up and to validate single and mn-PCR assays using DNA from negative and positive controls (see materials and methods). Neither false negative nor false positive were found in the assays. Horses were considered positive for equine piroplasmids when at least one of the seven gene targets was amplified. A single infection were recorded when band profiles showed target genes only above or below bar-coding threshold (300 bp threshold cut-off). The profiles showing above or below bands signal *B. caballi* or *T. equi* infections, respectively. It was scored mixed infections when at least one target gene were simultaneously observed both below and above of the threshold. The PCR analysis for the 235 horse blood samples included in the survey showed that 72.8% of the blood samples (n = 171 horses) were positive for equine piroplasmids. The prevalence of infection for either piroplasmid alone was 66.0% (155 positive horses) for *T. equi* and 29.4% (69 positive horses) for *B. caballi*. Mixed infections with both *T. equi* and *B. caballi* were recorded for 22.6% of the samples (53 horses).

3.2. Analysis of amplification success of the designed primers

Focusing on the amplification results for known positive samples, the primers used for *T. equi* detection amplified DNA from the *β-tubulin* gene in 142 of 155 (91.6%) *T. equi*-positive samples, compared with 140 (90.3%) for the *cytB* gene and 137 (88.4%) for the 18S *rRNA* gene. Only 96 horses tested positive for *T. equi* based on the *EMA-1* gene target, and this gene target appeared in combination with the other three gene targets in 95 out of the 96 samples. Of the 155 total horses diagnosed as *T. equi*-positive, 95 had amplified DNA for all four gene targets, 30 for three of the targets, 15 for two targets and 15 for a single target.

The results for the 69 *B. caballi*-positive horses were as follows: fragments of the *RAP-1*, the 18S *rRNA* and the *β-tubulin* genes were amplified for 52 (75.4%), 46 (66.7%) and 41 (59.4%) horses, respectively. In this case, 26 of the positive animals had amplified DNA for all three gene targets, 18 for two gene targets and 25 for a single target.

To confirm that an unspecific ~250 bp band was not a duplicated fragment of the expected *EMA-1* gene fragment, the F-inner (18Sequi and EMA-1) or R-inner (GM-tub and cytB) primers (Table 1) for *T. equi* were FAM-labelled. Both controls and eight DNA samples from acute infected horses were amplified by mn-PCR. PCR products were visualized by capillary electrophoresis (Fig. 3) aimed at sizing the four fragments exactly and discarding the unspecified ~250 bp band. As a result, all the fragments were estimated with a deviation of 5 bp or less from the expected size (Table 1 and Fig. 3).

Table 2a
Presence of *T. equi* and *B. caballi* in horses from Spain, in relation to age and gender.

	Risk factor		cELISA		mn-PCR	
			T. equi (%)	*B. caballi* (%)	*T. equi* (%)	*B. caballi* (%)
Nº of positive (%)			145 (61.7)	9 (3.8)	155 (66.0)	69 (29.4)
	Age	< 2 years	32 (22.0)	4 (44.4)	41 (26.5)	36 (52.2)
		2-11 years	89 (61.4)	5 (55.6)	91 (58.7)	29 (42.0)
		> 12 years	24 (16.6)	0 (0.0)	23 (14.8)	4 (5.8)
		χ^2	95,97	1.01	78.82	3.81
		p-value	0.001	0.63	0.001	0.14
	Gender	Male	27 (18.6)	2 (22.2)	33 (21.3)	14 (20.3)
		Female	118 (81.4)	7 (77.8)	122 (78.7)	55 (79.7)
		χ^2	4.83	0.007	1.135	0.529
		p-value	0.039	0.93	0.33	0.50

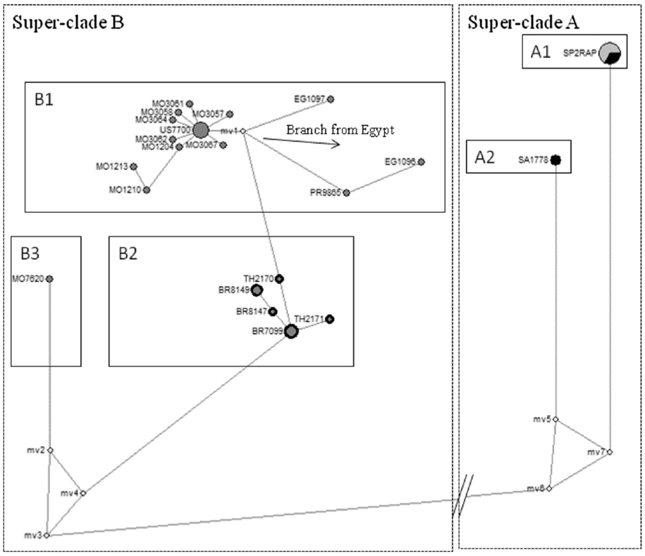

Fig. 4. Phylogenetic relationships among the *B. caballi* sequences of the *RAP-1*gene using the reduced median-joining Network (Bandelt et al., 1999). Large arrows signal super-clades: A for Afro-Mediterranean super-clade and B for Asia-American super clade. A1 signals represent Iberian (black portion) and Israeli (gray portion) Mediterranean lineage and A2 African lineage. B1 regroups Mongolian and USA sequences, and includes the Egyptian branch, B2 for tropical sequences and B3 includes an isolated from Mongolia. MO, US, EG, PR, BR, TH, SP, SA refer to Mongolia, USA, Egypt, Puerto Rico, Brazil, Thailand, Spain and South Africa, respectively; the numbers referring to the last four digits of Acc. Numbers. Additional information is summarized in table S2.

3.3. Phylogenetic analysis of the B. caballi RAP-1 gene

Due to poor results reported for the diagnosis of *B. caballi* by cELISA (Montes et al., 2017b), we conducted a genetic study with the *RAP-1* gene using sequences from different *B. caballi* strains (Fig. 4). The sequences covered 769 bp of the *RAP-1* gene (accession numbers MH538120 to MH 538122). After submitting to BLAST analysis, the three Spanish sequences showed 100% identity to the *RAP-1* gene from Israeli isolates (accession numbers: KF059875 to KF059877 and KF059879. After truncating to 358 bp (expanding from nucleotide 290 to 647 in the complete *RAP-1* sequence, AF092736) we still recorded a strong phylogenetic signal representing defined geographic clades based on Median-Joining Network analysis. In the network, two separated super-clades were shown at a global scale. The A super-clade regrouped sequences from African and Mediterranean *B. caballi* isolates (represented as A in Fig. 4) which later split into two discrete lineages; A1 with sequences geographically located in the Mediterranean basin and A2 mainly found in Africa. Super-clade B included a larger number of sequences from northern Asia and South America which split into three lineages (B1 to B3, Fig. 4). Lineage B1 showed a more star-like configuration, being highly frequent in Northern Asia and North America but surprisingly with a radiation branch in Egypt; the B2 lineage was geographically located in tropical areas of Asia and South America and B3 was a distinct lineage from Mongolia (B3).

3.4. Serological analysis

Of the 235 horse blood samples examined serologically, 62.6% (147 positive horses) were positive for equine piroplasmids. The seroprevalance for either piroplasmid separately was 61.7% (145 horses) for *T. equi* but only 3.8% for *B. caballi* (9 horses) (Table 2a). Seven horses were found to have antibodies against both piroplasmids, indicating co-infection.

3.5. cELISA versus mn-PCR results

The statistical analysis indicated that neither cELISA nor mn-PCR positive results for *B. caballi* were associated with horse age or sex. Risk factors found to be associated with *T. equi* seropositivity (by cELISA) were age ($p = 0.001$) and sex ($p = 0.039$) but only age ($p = 0.001$) for mn-PCR results (Table 2a). However, logistic regression confirmed only horse age as a significant risk factor. Older horses were more likely to be positive for *T. equi* by mn-PCR or cELISA, but with our data we did not obtain a result for *B. caballi* as relevant as in the case of *T. equi* (Table 2b).

The diagnostic performances of the two methods used in the study (cELISA and mn-PCR) were compared simultaneously for equine piroplasmids by the McNemar Chi-Square test for two related data sets. These results confirmed that the mn-PCR technique detected significantly higher infection prevalence of equine piroplasmids compared with the cELISA in apparently healthy horses ($\chi^2 = 18.581$, df = 1, $p < 0.0001$). Comparison for each parasite separately (*i.e.*, the cELISA *vs.* mn-PCR for *B. caballi* and the cELISA *vs.* mn-PCR for *T. equi*) showed similar results (*p*-value = 0.0001 and 0.031, respectively).

Table 2b
Logistic regression analysis including the OR of potential risk factors associated with *T. equi* and *B. caballi* with respect to serological and nm-PCR positivity.

		Risk factor		b	*p-value*	OR	95% CI
T. equi	cELISA	Age	< 2 years	*	*	*	*
			2-11 years	3.255	0.001	25.911	11.134 – 60.301
			> 12 years	2.660	0.001	14.303	4.588 – 44.594
		Gender	Male	*	*	*	*
			Female	−0.256	0.518	0.774	0.357 - 1.681
	mn-PCR	Age	< 2 years	*	*	*	*
			2-11 years	−2.031	0.001	27.155	10.981 – 67.153
			> 12 years	1.270	0.001	7.623	2.664 – 21.814
		Gender	Male	*	*	*	*
			Female	−0.671	0.077	0.511	0.243 - 1.074
B. caballi	cELISA	Age	< 2 years	*	*	*	*
			2-11 years	0.456	0.552	1.578	0.350 -7.108
			> 12 years	−17.956	0.998	0.000	0.000
		Gender	Male	*	*	*	*
			Female	−0.323	0.725	0.724	0.119 - 4.388
	mn-PCR	Age	< 2 years	*	*	*	*
			2-11 years	−0.238	0.460	0.788	0.419 - 1.482
			> 12 years	−1.070	0.064	0.343	0.110 - 1.065
		Gender	Male	*	*	*	*
			Female	0.252	0.507	1.287	0.610 - 2.714

(OR, Odds Ratio; 95% CI, 95% Confidence interval; * Reference category).

Table 3
Contingency table showing positive and negative comparisons of proportions for (A) cELISA-cab *vs* mn-PCR-cab and (B) cELISA-equi *vs* mn-PCR-equi.

(A)		mn-PCR-cab		
		Negative (%)	Positive (%)	Total
cELISA-cab	Negative	68.9	27.2	96.2
	Positive	1.7	2.1	3.8
	Total	70.6	29.4	100
(B)		**mn-PCR-equi**		
		Negative (%)	Positive (%)	Total
cELISA-equi	Negative	32.3	6.0	38.3
	Positive	1.7	60.0	61.7
	Total	34.0	66.0	100.0

Consequently, the mn-PCR method could more efficiently detect carrier horses, considering that sampled horses were apparently healthy. Also, the concordance between both the serological and the mn-PCR methods for *T. equi* and *B. caballi*, separately, was assessed using the Kappa´s coefficient. The pair cELISA *vs.* mn-PCR in latent *T. equi* infection reached a highly significant agreement index (0.83 ± 0.037 SE; p = 0.0001) but for the cELISA *vs. B. caballi* mn-PCR the null hypothesis was not rejected which indicates disagreement (0.06 ± 0.040 SE; p = 0.13) (Table 3).

4. Discussion

4.1. The multinested PCR application

Previous studies showed nested PCR (Nicolaiewsky et al., 2001; Rampersad et al., 2003; Baldani et al., 2008) and multiplex PCR (Alhassan et al., 2005) to increase sensitivity and specificity over conventional single PCR for detecting equine piroplasmids. The choice of target genes was critically related to the success of the PCR assays (Alhassan et al., 2007), but the combination of the two PCR variants (multiplex and nested PCR) and the development of a set of primers (derived from informative sequences) improved EP diagnosis. In contrast to Alhassan et al. (2005), who only used one gene (*18S rRNA*), the use of mn-PCR based on four different genes and seven primer pairs (some of them species-specific) allowed for the simultaneous detection of *T. equi* and *B. caballi*. Three genes (the *β-tubulin* gene, the *cytB* gene and the *18S rRNA* gene) seemed to be appropriate markers to detect *T. equi* as each one appeared in around 90% of the positive bloods. Meanwhile, the *EMA-1* gene allowed diagnosis only in 61.9% of the cases. The existence of different *EMA* gene sequences (Bhoora et al., 2010c; Baptista et al., 2013) or because the *EMA-1* is a single-copy gene (Knowles et al., 1997; Ueti et al., 2003) has been suggested to be the reason why it was less efficient than multiple-copy genes (*e.g.*, *cytB* gene).

In the present study, it has been demonstrated that the overall EP prevalence by mn-PCR (72.8%) was higher than those obtained in other countries using different PCR variants (Qablan et al., 2013; Bahrami et al., 2014; Gallusová et al., 2014; Malekifard et al., 2014; Sumbria et al., 2016; Guven et al., 2017), although molecular differences among lineages of both parasites may explain these discrepancies. Furthermore, the *T. equi* prevalence was higher than for *B. caballi*. Similar results were obtained using different diagnostic methods (serological or molecular) (reviewed by Montes et al., 2017b). The higher prevalence detected in this study for *B. caballi* using the mn-PCR in comparison with the cELISA, could be attributed to the fact that the mn-PCR may be more specific for detecting the horses infected with the *B. caballi* parasite.

In addition, PCR assays based on detection of superficial proteins encoded by paralogous single-copy genes for *T. equi* (Ueti et al., 2003) and rhoptria-proteins encoded by a gene containing more than two copies for *B. caballi* genome might negatively impact the sensitivity and specificity of single-PCR because diversity or even mutations are expected on different isolates (Heim et al., 2007). Thus, procedures as the multiplex-PCR can be designed to overcome this technical drawback for EP detection, although to be sure of the success of this method it would be advisable to use all the available molecular information as it has been done in this study.

4.2. Genetic structure of B. caballi based on the RAP-1 gene

The network revealed strong phylogeographic structure due to major branching events, suggesting ancient genetic isolation of lineages, later accompanied by regional expansions. Moreover, at least two factors may influence the geographical distribution of *B. caballi* super-clade: the migratory behaviour of equine species and the presence in the area of the appropriate vector. Unfortunately, neither issue has been explored in depth yet. Past and recent worldwide movements of horses should also be considered. The signature of demographic expansion was more evident for the B super-clade considering its more star-like network structure (Bandelt et al., 1999) in Mongolia/USA and Tropical sub-clade, but not so for the A super-clade. This could be related to the few studies regarding the A super-clade or the reduction of the molecular information after trimming the *RAP-1* gene. Other studies also found obvious genetic diversity in sequences from different countries (Bhoora et al., 2010b; Munkhjargal et al., 2013; Rapoport et al., 2014) and according to Munkhjargal et al. (2013), the sequences from Mongolia and America cluster together.

4.3. Seroprevalence of equine piroplasmids

The overall seroprevalence of equine piroplasmids based on cELISA was higher than observed in other studies from Spain (García-Bocanegra et al., 2013; Camacho et al., 2005; Habela et al., 2005; Montes et al., 2017b; Camino et al., 2018),which may be due to geographical variations, differences in horse management practices and tick control measures.

4.4. ELISA versus mn-PCR

The higher prevalence observed by mn-PCR supports the existence of differences in efficiency between this method and the commercial cELISA kits for *T. equi*, as it was reported by Mahdy et al. (2016), who compared the nPCR with the cELISA. Focusing on *B. caballi*, Bhoora et al. (2010b) and Rapoport et al. (2014) in South Africa and Israel, respectively, showed that the IFAT and the PCR were more efficient than commercial cELISA kits. The genotypes analysed by these authors belonged to the A super-clade, as did the sequences from Spain of this study. In contrast, these kits were quite successful in the detection of *B. caballi* in other countries (Rosales et al., 2013; Mahmoud et al., 2016). The reason for this may be that *B. caballi* genotypes from those countries includes American/Caribbean sequences belonging to the B super-clade (this study) and whose *RAP-1* gene has been used to prepare the recombinant antigen of the commercial cELISA kits. However, other authors have indicated that the PCR analysis could be less efficient than serological tests (Teglas et al., 2005; Heim et al., 2007; Motloang et al., 2008; Salim et al., 2008; Moretti et al., 2010; Grandi et al., 2011; Laus et al., 2013; Abedi et al., 2014; Bartolomé del Pino et al., 2016). Therefore, the diversity of results showed in different countries seems to support the idea that the genetic diversity of these parasites might be hindering a more successful detection of EP. In accordance with Bhoora et al. (2010b), redesigning the current cELISA kit using a conserved epitope of the *RAP-1* antigen may overcome this problem.

The mn-PCR did not recognise DNA of *T. equi* in 4 out of the 145 seropositive sera which suggests parasite clearance from the circulating blood (Allsopp et al., 2007; Bhoora et al., 2010a; Baptista et al., 2013; Sumbria et al., 2016). Nevertheless, 14 animals were mn-PCR positive and cELISA negative for *T. equi*, which could be explained by the possible genetic differences between the sequences of the genes used to design the recombinant proteins in commercial kits (*EMA-1* and *RAP-1*) and the strains from different countries. In fact, Bhoora et al. (2010c) reported that the heterogeneity of the sequences of the *EMA-1* gene from *T. equi* were related to differences in the prevalence obtained by PCR. On the other hand, the mn-PCR did not amplify *B. caballi* DNA in 4 seropositive animals. Some factors could explain this; for instance, that the *B. caballi* parasite would be present in blood circulation in a low parasitaemia (Potgieter et al., 1992; Holman et al., 1993) or the parasites could have been eliminated completely from the animal. Additionally, 64 horses were found positive with the mn-PCR and negative with the cELISA. Thus, the mn-PCR may be more effective than the cELISA, therefore, the antigens of this commercial kit are less specific for genotypes from Spain and one of the reasons why Laus et al. (2013) and Sgorbini et al. (2015) strongly recommend the use of both diagnostic techniques.

Relating to the statistical results, some authors have obtained a similar κ value than that observed in the present study for *T. equi* when using the conventional PCR and the cELISA (Farkas et al., 2013). Lobanov et al. (2018), who compared a duplex qPCR with the commercial cELISA kit, demonstrated that there was a good agreement ($\kappa = 0.620$) for *T. equi*, but lower than that observed in this study (mn-PCR *vs*. cELISA). However, the agreement for *B. caballi* ($\kappa = 0.127$) in Lobanov et al. (2018) was similar to our study (slight agreement, in accordance with Landis and Koch, 1977). However, this similarity is misleading as the cELISA kit works better with the samples from Brazil (Lobanov et al., 2018) but the mn-PCR works better with the Spanish samples. In any case, these studies showed better agreement for *T. equi* than for *B. caballi*. Focusing on risk factors associated to EP (horse sex and age), the results that Sumbria et al. (2016) found, were similar to us for *T. equi* but not for *B. caballi* by cELISA. García-Bocanegra et al. (2013) and Davitkov et al. (2016) concluded with the same results as the present study for *B. caballi* but not for *T. equi* by cELISA and multiplex PCR, respectively. The specific results for *T. equi* were diverse, *e.g.* Ayala-Valdovinos et al. (2017) by nPCR, showed similar results to those in this study. Moreover, the OR would confirm that 2–11 year old animals are more likely to be detected as parasitized for *T. equi* as other authors reported (Rüegg et al., 2007; Kouam et al., 2010; Cantú-Martínez et al., 2012; Montes et al., 2017b, this study). Several authors (Mujica et al., 2011; Abutarbush et al., 2012; Farkas et al.,2013; Munkhjargal et al., 2013; Ribeiro et al., 2013; Malekifard et al.,2014; Kizilarslan et al., 2015; Posada-Guzmán et al., 2015; Dahiya et al., 2018; Ebrahimi et al., 2018) using the serological tests and/or the PCR determined that neither sex nor age were risk factors. In general, the statistical analysis carried out in the present study indicates that sex is not revealed as a risk factor associated to the presence of EP. Some reasons could explain this, for instance, the management practices carried out in the herds of horses, since most of the young animals are raised in the field and they are exposed to ticks or the unbalanced distribution of the number of samples of both sexes groups. Regarding age, we concluded that it was a risk factor only for *T. equi*, not for *B. caballi*. This could be explained because *T. equi* does not disappear from the bloodstream of the animal (Brüning, 1996) or due to reinfections, increasing the number of positive animals as they become older (Montes et al., 2017b). However, the *B. caballi* parasite tends to be eliminated from the blood after 1–4 years post-infection (De Waal, 1992).

5. Conclusions

Due to the robust diagnosis of horses carrying EP (mainly for *B. caballi* infections), the mn-PCR assays seem to be a promising technique for global EP control, after the comparison with the cELISA. This fact has been demonstrated in the present study.

In addition, a source of new validated primer pairs that simultaneously amplified different species-specific genes was provided, contributing to increase the diagnostic usage of this technique. The framework of this study is relevant worldwide because endemic regions such as Spain need reliable molecular diagnostic methods to address horse trade both inside and outside their borders, and to mitigate problems with international trade of Spanish autochthonous animals. Also, the ample genetic diversity found for the *RAP-1* gene may explain part of the variation that might influence the antigenic characteristic of this species (*B. caballi*) and therefore, may be among the reasons for the low agreement between the cELISA and the mn-PCR for this parasite.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2018.11.008.

References

Abedi, V., Razmi, G., Seifi, H., Naghibi, A., 2014. Molecular and serological detection of *Theileria equi* and *Babesia caballi* in horses and ixodid ticks in Iran. Ticks Tick. Dis. 5, 239–244. https://doi.org/10.1016/j.ttbdis.2013.11.008.

Abutarbush, S.M., Alqawasmeh, D.M., Mukbel, R.M., Al-Majali, A.M., 2012. Equine babesiosis: seroprevalence, risk factors and comparison of different diagnostic methods in Jordan. Transbound. Emerg. Dis. 59, 72–78. https://doi.org/10.1111/j.1865-1682.2011.01244.x.

Alhassan, A., Pumidonming, W., Okamura, M., Hirata, H., Battsetseg, B., Fujisaki, K., Yokoyama, N., Igarashi, I., 2005. Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. Vet. Parasitol. 129, 43–49. https://doi.org/10.1016/j.vetpar.2004.12.018.

Alhassan, A., Iseki, H., Kim, C., Yokoyama, N., Igarashi, I., 2007. Comparison of polymerase chain reaction methods for the detection of *Theileria equi* infection using whole blood compared with pre-extracted DNA samples as PCR templates. Trop. Anim. Health Prod. 39, 369–374. https://doi.org/10.1007/s11250-007-9025-1.

Allsopp, M.T.E.P., Lewis, B.D., Penzhorn, B.L., 2007. Molecular evidence for transplacental transmission of *Theileria equi* from carrier mares to their apparently healthy foals. Vet. Parasitol. 148, 130–136. https://doi.org/10.1016/j.vetpar.2007.05.017.

Ayala-Valdovinos, M.A., Lemus-Flores, C., Galindo-García, J., Sánchez-Chipres, D., Duifhuis-Rivera, T., Anguiano-Estrella, R., Bañuelos-Pineda, J., Rodríguez-Carpena, J.G., 2014. Nested PCR detection of *Theileria equi* infection and frequency in horses imported into Mexico. J. Anim. Vet. Adv. 13, 859–863. https://doi.org/10.3923/javaa.2014.859.863.

Ayala-Valdovinos, M.A., Lemus-Flores, C., Galindo-García, J., Bañuelos-Pineda, J., Rodríguez-Carpena, J.G., Sánchez-Chipres, D., Duifhuis-Rivera, T., 2017. Diagnosis and prevalence of *Theileria equi* in western Mexico by nested PCR. Parasitol. Int. 66, 821–824. https://doi.org/10.1016/j.parint.2016.09.011.

Bahrami, S., Ghardrdan, A.R., Mirabdollahi, S.M., Fayed, M.R., 2014. Diagnosis of subclinical equine theileriosis in center of Iran using parasitological and molecular methods. Trop. Biomed. 31, 110–117. https://doi.org/10.1590/S0102-09352008000300005.

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48.

Baptista, C., Lopes, M.S., Tavares, A.C., Rojer, H., Kappmeyer, L., Mendoça, D., da Câmara Machado, A., 2013. Diagnosis of *Theileria equi* infections in horses in the Azores using cELISA and nested PCR. Ticks Tick. Dis. 4, 242–245. https://doi.org/10.1016/j.ttbdis.2012.11.008.

Bartolomé del Pino, L.E., Nardini, R., Veneziano, V., Iacoponi, F., Cersini, A., Autorino, G.L., Buono, F., Sicluna, M.T., 2016. *Babesia caballi* and *Theileria equi* infections in horses in Central-Southern Italy: Sero-molecular survey and associated risk factors. Ticks Tick. Dis. 7, 462–469. https://doi.org/10.1016/j.ttbdis.2016.01.011.

Bashiruddin, J.B., Cammâ, C., Rebêlo, E., 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. Vet. Parasitol. 84, 75–83. https://doi.org/10.1016/S0304-4017(99)00049-7.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., Van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2010a. Development and evaluation or real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Vet. Parasitol. 168, 201–211. https://doi.org/10.1016/j.vetpar.2009.11.011.

Bhoora, R., Quan, M., Zweygarth, E., Guthrie, A.J., Prinsloo, S.A., Collins, N.E., 2010b. Sequence heterogeneity in the gene encoding the rhoptry-associated protein-1 (RAP-1) of *Babesia caballi* isolates from South Africa. Vet. Parasitol. 169, 279–288. https://doi.org/10.1016/j.vetpar.2010.01.009.

Bhoora, R., Quan, M., Matjila, P.T., Zweygarth, E., Guthrie, A.J., Collins, N.E., 2010c. Sequence heterogeneity in the equi merozoite antigen gene (ema-1) of *Theileria equi* and development of an ema-1-specific TaqMan MGB assay for the detection of *T. Equi*. Vet. Parasitol. 172, 33–45. https://doi.org/10.1016/j.vetpar.2010.04.025.

Böse, R., Jorgesen, W.K., Dalgliesh, R.J., Friedhoff, K.T., de Vos, A.J., 1995. Current state and future trends in the diagnosis of babesiosis. Vet. Parasitol. 57, 61–74. https://doi.org/10.1016/0304-4017(94)03111-9.

Brüning, A., 1996. Equine piroplasmosis: an update on diagnosis, treatment and prevention. Br. Vet. J. 152, 139–151.

Brüning, A., Phipps, P., Posnett, E., Canning, E.U., 1997. Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. Vet. Parasitol. 68, 11–26.

Cacciò, S., Cammà, C., Onuma, M., Severini, C., 2000. The *β-tubulin* gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. Int. J. Parasitol. 30, 1181–1185. https://doi.org/10.1016/S0020-7519(00)00105-3.

Camacho, A.T., Guitian, F.J., Pallas, E., Gestal, J.J., Olmeda, A.S., Habela, M.A., Telford III, S.R., Spielman, A., 2005. *Theileria* (*Babesia*) *equi* and *Babesia caballi* Infections in Horses in Galicia. Spain. Trop. Anim. Health Prod. 37, 293–302. https://doi.org/10.1007/s11250-005-5691-z.

Camino, E., de la Cruz, M.L., Dominguez, L., Carvajal, K.A., Fores, P., de Juan, L., Cruz-Lopez, F., 2018. Epidemiological situation of the exposure to agents causing equine piroplasmosis in Spanish purebred horses in Spain: seroprevalence and associated risk factors. J. Equine Vet. Sci. 67, 81–86. https://doi.org/10.1016/j.jevs.2018.03.012.

Cantú-Martínez, M.A., Segura-Correa, J.C., Silva-Páez, M.L., Avalos-Ramírez, R., Wagner, G.G., 2012. Prevalence of antibodies to *Theileria equi* and *Babesia caballi* in horses from Northeastern Mexico. J. Parasitol. 98, 869–870. https://doi.org/10.1645/GE-3064.1.

Dahiya, R., Kumar-Salar, R., Mandal, K.D., Kumar, R., Tripathi, B.N., Pal, Y., Kumar, S., 2018. Risk factors analysis associated with *Theileria equi* infected equines in semi-arid and sub-humid ecological enzootic zones of India. Vet. Parasitol. Reg. Stud. Rep. 12, 17–21. https://doi.org/10.1016/j.vprsr.2018.01.005.

Davitkov, D., Vucicevic, M., Stevanovic, J., Krstic, V., Slijepcevic, D., Glavinic, U., Stanimirovic, Z., 2016. Molecular detection and prevalence of *Theileria equi* and *Babesia caballi* in horses of central Balkan. Acta Parasitol. 61, 337–342. https://doi.org/10.1515/ap-2016-0044.

De Waal, D.T., 1992. Equine piroplasmosis: a review. Br. Vet. J. 148, 6–14. https://doi.org/10.1016/0007-1935(92)90061-5.

Ebrahimi, M., Adinehbeigi, K., Hamidinejat, H., Tabandeh, M.R., 2018. Molecular characterization of *Theileria equi* infection in horse populations belonging to West Azerbaijan, Iran: insights into the importance of Equine Merozoite Antigen (EMA-1) in its diagnosis. Ann. Parasitol. 64, 21–27. https://doi.org/10.17420/ap6401.128.

Farkas, R., Tanczos, B., Gyurkovszky, M., Fölsvári, G., Solymosi, N., Edelhofer, R., Hornok, S., 2013. Serological and molecular detection of *Theileria equi* infection in horses in Hungary. Vet. Parasitol. 192, 143–148. https://doi.org/10.1016/j.vetpar.2012.09.035.

Fernández-García, J.L., 2012. The endangered *Dama dama mesopotamica*: genetic variability, allelic loss and hybridization signals. Contrib. Zool. 81, 223–233.

Ferreira, E.P., Vidotto, O., Almeida, J.C., Ribeiro, L.P., Borges, M.V., Pequeno, W.H., Stipp, D.T., de Oliveira, C.J., Biondo, A.W., Vieira, T.S., Vieira, R.F., 2016. Serological and molecular detection of *Theileria equi* in sport horses of northeastern Brazil. Comp. Immunol. Microbiol. Infect. Dis. 47, 72–76. https://doi.org/10.1016/j.cimid.2016.06.004.

Forster, P., Torroni, A., Renfrew, C., Röhl, A., 2001. Phylogenetic star contraction applied to Asian and Papuan mtDNA evolution. Mol. Biol. Evol. 18, 1864–1881.

Friedhoff, K.T., Soulé, C., 1996. An account on equine babesiosis. Rev. Sci. Tech. 15, 1191–1201. https://doi.org/10.20506/rst.15.3.972.

Gallusová, M., Qablan, M.A., D'Amico, G., Oborník, M., Petrželková, K.J., Mihalca, A.D., Modrý, D., 2014. Piroplasms in feral and domestic equines in rural areas of the Danube Delta, romania, with survey of dogs as a possible reservoir. Vet. Parasitol. 206, 287–292. https://doi.org/10.1016/j.vetpar.2014.10.018.

García-Bocanegra, I., Arenas-Montes, A., Hernández, E., Adaszek, L., Carbonero, A., Almería, S., Jaén-Téllez, J.A., Gutiérrez-Palomino, P., Arenas, A., 2013. Seroprevalence and risk factors associated with *Babesia caballi* and *Theileria equi* infection in equids. Vet. J. 195 (2), 172–178. https://doi.org/10.1016/j.tvjl.2012.06.012.

Grandi, G., Molinari, G., Tittarelli, M., Sassera, D., Kramer, L.H., 2011. Prevalence of *Theileria equi* and *Babesia caballi* infection in horses from Northern Italy. Vector Borne Zoonotic Dis. 11, 955–956. https://doi.org/10.1089/vbz.2010.0193.

Guven, E., Avcioglu, H., Deniz, A., Balkaya, İ., Abay, U., Yavuz, Ş., Akyüz, M., 2017. Prevalence and molecular characterization of *Theileria equi* and *Babesia caballi* in jereed horses in Erzurum. Turkey. Acta Parasitol. 62 (1), 207–213. https://doi.org/10.1515/ap-2017-0025.

Habela, M.A., Gragera-Slikker, A., Moreno, A., Montes, G., Sevilla, R., 2005. Piroplasmosis equina: conocimiento y grado de concienciación de los productores de caballos Pura Raza Española. Revista Equinus 11, 17–34.

Heim, A., Passos, L.M.F., Ribeiro, M.F.B., Costa-Júnior, L.M., Bastos, C.V., Cabral, D.D., Hirzmann, J., Pfister, K., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. Parasitol. Res. 102, 63–68. https://doi.org/10.1007/s00436-007-0726-1.

Holman, P.J., Frerichs, W.M., Chieves, L., Wagner, G.G., 1993. Culture confirmation of the carrier status of *Babesia caballi*-infected horses. J. Clin. Microbiol. 31, 698–701.

Kappmeyer, L.S., Perryman, L.E., Hines, S.A., Baszler, T.V., Katz, J.B., Hennager, S.G., Knowles, D.P., 1999. Detection of equine antibodies to Babesia caballi by recombinant B. caballi rhoptry-associated protein 1 in a competitive-inhibition enzyme-linked immunosorbent assay. J. Clin. Microbiol. 37, 2.285–2.290.

Kim, C., Conza, L.B., Alhassan, A., Iseki, H., Yokoyama, N., Xuan, X., Igarashi, I., 2008. Diagnostic real-time PCR assay for the quantitative detection of *Theileria equi* from equine blood samples. Vet. Parasitol. 151, 158–163. https://doi.org/10.1016/j.vetpar.2007.10.023.

Kizilarslan, F., Yildirim, A., Duzlu, O., Inci, A., Onder, Z., Ciloglu, A., 2015. Molecular detection and characterization of *Theileria equi* and *Babesia caballi* in horses (*Equus ferus caballus*) in Turkey. J. Equine Vet. Sci. 35, 830–835. https://doi.org/10.1016/j.jevs.2015.08.002.

Knowles, D.P., Perryman, L.E., Kappmeyer, L.S., Hennager, S.G., 1991. Detection of equine antibody to *Babesia equi* merozoite proteins by a monoclonal antibody-based competitive inhibition enzyme-linked inmunosorbent assay. J. Clin. Microbiol. 29, 2056–2058.

Knowles, D.P., Kappmeyer, L.S., Stiller, D., Hennager, S.G., Perryman, L.E., 1992. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*. J. Clin. Microbiol. 12, 3122–3126.

Knowles, D.P., Kappmeyer, L.S., Perryman, L.E., 1997. Genetic and biochemical analysis of erythrocyte-stage surface antigens belonging to a family of highly conserved proteins of *Babesia equi* and *Theileria* species. Mol. Biochem. Parasitol. 90, 69–79.

Kouam, M.K., Kantzoura, V., Gajadhar, A.A., Theis, J.H., Papadopoulos, E., Theodoropoulos, G., 2010. Seroprevalence of equine piroplasms and host-related factors associated with infection in Greece. Vet. Parasitol. 169, 273–278. https://doi.org/10.1016/j.vetpar.2010.01.011.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159–174. https://doi.org/10.2307/2529310.

Laus, F., Veronesi, F., Passamonti, F., Paggi, E., Cerquetella, M., Hyatt, D., Tesei, B., Fioretti, D.P., 2013. Prevalence of tick borne pathogens in horses from Italy. J. Vet. Med. Sci. 75, 715–720. https://doi.org/10.1292/jvms.12-0449.

Lobanov, V.A., Peckle, M., Massard, C.L., Scandrett, W.B., Gajadhar, A.A., 2018. Development and validation of a duplex real-time PCR assay for the diagnosis of equine piroplasmosis. Parasit. Vectors 11 (125). https://doi.org/10.1186/s13071-018-2751-6.

Mahdy, O.A., Nassar, A.M., Mohamed, B.S., Mahmoud, M.S., 2016. Comparative diagnosis utilizing molecular and serological techniques of *Theileria equi* infection in distinct equine populations in Egypt. Int. J. Chem. Tech. Res. 9, 185–197.

Mahmoud, M., El-Ezz, N.T., Abdel-Shafy, S., Nassar, S.A., El Namaky, A.H., Khalil, W.K., Knowles, D., Kappmeyer, L., Silva, M.G., Suarez, C.E., 2016. Assessment of *Theileria equi* and *Babesia caballi* infections in equine populations in Egypt by molecular, serological and haematological approaches. Parasit. Vectors 9 (260). https://doi.org/10.1186/s13071-016-1539-9.

Malekifard, F., Tavassoli, M., Yakhchali, M., Darvishzadeh, R., 2014. Detection of *Theileria equi* and *Babesia caballi* using microscopic and molecular methods in horses in suburb of Urmia. Iran. Vet. Res. Forum. 5, 129–133.

Mans, B.J., Pienaar, R., Latif, A.A., 2015. A review of *Theileria* diagnostics and epidemiology. Int. J. Parasitol. Parasites Wildl. 4, 104–118. https://doi.org/10.1016/j.ijppaw.2014.12.006.

Mehlhorn, H., Schein, E., 1998. Redescription of Babesia equi Laveran, 1901 as Theileria equi. Parasitol Res. 84 (6), 467–475. https://doi.org/10.1007/s004360050431.

Millar, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215. https://doi.org/10.1093/nar/16.3.1215.

Montes, M.G., Fernández-García, J.L., Habela, M.A., 2017a. Genetic variation of the *β-tubulin* gene of *Babesia caballi* strains. J. Arthropod. Dis. 11, 344–353.

Montes, M.G., Fernández-García, J.L., Habela, M.A., 2017b. Seroprevalence of *Theileria equi* and *Babesia caballi* in horses in Spain. Parasite 24, 14. https://doi.org/10.1051/parasite/2017015.

Moretti, A., Mangili, V., Salvatori, R., Maresca, C., Scoccia, E., Torina, A., Moretta, I., Gabrielli, S., Tampieri, M.P., Pietrobelli, M., 2010. Prevalence and diagnosis of *Babesia* and *Theileria* infections in horses in Italy: a preliminary study. Vet. J. 184, 346–350. https://doi.org/10.1016/j.tvjl.2009.03.021.

Motloang, M.Y., Thekisoe, O.M., Alhassan, A., Bakheit, M., Motheo, M.P., Masangane, F.E., Thibedi, M.L., Inoue, N., Igarashi, I., Sugimoto, C., Mbati, P.A., 2008. Prevalence of *Theileria equi* and *Babesia caballi* infections in horses belonging to resource-poor farmers in the north-eastern Free State Province, South Africa. Onderstepoort J. Vet. Res. 75, 141–146. https://doi.org/10.4102/ojvr.v75i2.12.

Mujica, F.F., Perrone, T., Forlano, M., Coronado, A., Meléndez, R.D., Barrios, N., Álvarez, R., Granda, F., 2011. Serological prevalence of *Babesia caballi* and *Theileria equi* in horses of Lara state. Venezuela. Vet. Parasitol. 178, 180–183. https://doi.org/10.1016/j.vetpar.2010.12.036.

Munkhjargal, T., Sivakumar, T., Battsetseg, B., Nyamjargal, T., Aboulaila, M., Purevtseren, B., Bayarsaikhan, D., Byambaa, B., Terkawi, M.A., Yokoyama, N., Igarashi, I., 2013. Prevalence and genetic diversity of equine piroplasms in Tov province. Mongolia. Infect. Genet. Evol. 16, 178–185.

Nicolaiewsky, T.B., Richter, M.F., Lunge, V.R., Cuhna, C.W., Delagostin, O., Ikuta, N., Fonseca, A.S., Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Vet. Parasitol. 101, 9–21.

Nuttall, G.H.F., Strickland, C., 1910. Die Parasiten der Pferdepiroplasmose des biliary fever. Zentralbl Bakteriol Mikrobiol Hyg [A] 56, 524–525.

OIE, 2017. Equine Piroplasmosis. Manual of Diagnostic Test and Vaccines for Terrestrial Animals. (accessed 22 September 2017) (Chapter 12.7). http://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_equine_piroplasmosis.pdf.

Polzin, T., Daneschmand, S.V., 2003. On Steiner trees and minimum spanning trees in hypergraphs. Oper. Res. Lett. 31, 12–20.

Posada, D., Crandall, K.A., 2001. Intraspecific gene genealogies: trees grafting into networks. Trends Ecol. Evol. (Amst.) 16, 37–45.

Posada-Guzmán, M.F., Dolz, G., Romero-Zúñiga, J.J., Jiménez-Rocha, A.E., 2015. Detection of *Babesia caballi* and *Theileria equi* in blood from equines from four indigenous communities in Costa rica. Vet. Med. Int. 2015, 236278. https://doi.org/10.1155/2015/236278.

Posnett, E.S., Ambrosio, R.E., 1991. DNA probes for the detection of Babesia caballi. Parasitology 102, 357–365. https://doi.org/10.1017/S0031182000064301.

Posnett, E.S., Fehrsen, J., de Waal, D.T., Ambrosio, R.E., 1991. Detection of *Babesia equi* in infected horses and carrier animals using a DNA probe. Vet. Parasitol. 39, 19–32. https://doi.org/10.1016/0304-4017(91)90058-4.

Potgieter, F.T., de Waal, D.T., Posnett, E.S., 1992. Transmission and diagnosis of equine babesiosis in South Africa. Mem. Inst. Oswaldo Cruz 87, 139–142.

Qablan, M.A., Obroník, M., Petrželková, K.J., Sloboda, M., Shudiefat, M.F., Hořín, P., Lukeš, J., Modrý, D., 2013. Infections by *Babesia caballi* and *Theileria equi* in Jordanian equids: epidemiology and genetic diversity. Parasitology 140, 1096–1103. https://doi.org/10.1017/S0031182013000486.

Rampersad, J., Cesar, E., Campbell, M.D., Samlal, M., Ammons, D., 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. Vet. Parasitol. 114, 81–87. https://doi.org/10.1016/S0304-4017(03)00129-8.

Rapoport, A., Aharonson-Raz, K., Berlin, D., Tal, S., Gottlieb, Y., Klement, E., Steinman, A., 2014. Molecular characterization of the *Babesia caballi* rap-1 gene and epidemiological survey in horses in Israel. Infect. Genet. Evol. 23, 115–120. https://doi.org/10.1016/j.meegid.2014.01.033.

Ribeiro, A.J., Cardoso, L., Maia, J.M., Coutinho, T., Cotovio, M., 2013. Prevalence of *Theileria equi*, *Babesia caballi* and *Anaplasma phagocytophilum* in horses from the north of Portugal. Parasitol. Res. 112, 2611–2617. https://doi.org/10.1007/s00436-013-3429-9.

Rosales, R., Rangel-Rivas, A., Escalona, A., Jordan, L.S., Gonzatti, M.I., Asom, P.M., Perrone, T., Silva-Iturriza, A., Mijares, A., 2013. Detection of *Theileria equi* and *Babesia caballi* infections in Venezuelan horses using Competitive-Inhibition ELISA and PCR. Vet. Parasitol. 196, 37–43. https://doi.org/10.1016/j.vetpar.2013.02.004.

Rüegg, S.R., Torgerson, P., Deplazes, P., Mathis, A., 2007. Age-dependent dynamics of *Theileria equi* and *Babesia caballi* infections in Southwest Mongolia based on IFAT and/or PCR prevalence data from domestic horses and ticks. Parasitology 134, 939–947. https://doi.org/10.1017/S0031182007002405.

Salim, B.O.M., Hassan, S.M., Bakheit, M.A., Alhassan, A., Igarashi, I., Karanis, P., Abdelrahman, M.B., 2008. Diagnosis of *Babesia caballi* and *Theileria equi* infections in horses in Sudan using ELISA and PCR. Parasitol. Res. 103, 1145–1150. https://doi.org/10.1007/s00436-008-1108-z.

Sgorbini, M., Bonelli, F., Nardoni, S., Rocchigiani, G., Corazza, M., Mancianti, F., 2015. Seroprevalence and molecular analysis of *Babesia caballi* and *Theileria equi* from Central Italy during a 10-year period. J. Equine Vet. Sci. 35, 865–868. https://doi.org/10.1016/j.jevs.2015.08.011.

Sumbria, D., Singla, L.D., Sharma, A., 2016. *Theileria equi* and *Babesia caballi* infection in equines in Punjab, India: a serological and molecular survey. Trop. Anim. Health Prod. 48, 45–52. https://doi.org/10.1007/s11250-015-0917-1.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197.

Teglas, M., Matern, E., Lein, S., Foley, P., Mahan, S.M., Foley, J., 2005. Ticks and tick-borne disease in Guatemalan cattle and horses. Vet. Parasitol. 131, 119–127. https://doi.org/10.1016/j.vetpar.2005.04.033.

Ueti, M.W., Palmer, G.H., Kappmeyer, L.S., Scoles, G.A., Knowles, D.P., 2003. Expression of equi merozoite antigen 2 during development of *Babesia equi* in the midgut and salivary gland of the vector tick *Boophilus microplus*. J. Clin. Microbiol. 41, 5803–5809. https://doi.org/10.1128/JCM.41.12.5803-5809.2003.

Uilenberg, G., 2006. Babesia: a historical overview. Vet. Parasitol. 138, 3–10. https://doi.org/10.1016/j.vetpar.2006.01.035.

Xuan, X., Larsen, A., Ikadai, H., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2001. Expresión of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 39, 705–709. https://doi.org/10.1128/JCM.39.2.705-709.2001.

Xuan, X., Chahan, B., Huang, X., Yokohama, N., Makala, L.H., Igarashi, I., Fujisaki, K., Maruyama, S., Sakai, T., Mikami, T., 2002. Diagnosis of equine piroplasmosis in Xinjiang province of China by the enzyme-linked immunosorbent assays using recombinant antigens. Vet. Parasitol. 108, 179–182. https://doi.org/10.1016/S0304-4017(02)00193-0.