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Research paper

Serological, molecular and hematological diagnosis in horses with clinical suspicion of equine piroplasmosis: Pooling strengths

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ABSTRACT

Equine piroplasmosis (EP) is a tick-borne protozoan disease caused by *Theileria equi* and/or *Babesia caballi*. Clinical signs (fever, pale mucosal membranes, jaundice), anemia and hyperbilirubinemia have been associated with the disease. EP is widespread, has a significant economic impact on the equine industry and remains endemic in Spain. This study was carried out with samples belonging to 140 horses residing in Spain and showing common clinical signs of EP. A blood smear microscopic examination and a comparison between the different results obtained by competitive Enzyme-Linked Immunosorbent Assay (cELISA), real-time Polymerase Chain Reaction (PCR) and hematological and biochemical (direct and total bilirubin) screening were conducted. EP positivity rates by cELISA and PCR were 50.7% and 42.9%, respectively, whereas only 9% of the horses were positive in the microscopic analysis. A significantly higher number of *B. caballi*-positive horses were detected by cELISA than PCR, and Kappa value was higher for *T. equi* ($k = 0.575$) than for *B. caballi* ($k = 0.401$). For the first time, an association between a high ELISA inhibition percentage (IP) and a positive PCR result for *B. caballi* was determined. Although most authors have described *T. equi* as more pathogenic than *B. caballi*, we found that horses parasitized by *B. caballi* showed a more severe hemolytic anemia, whereas *T. equi* infections were mostly associated with leukocytosis. The hemogram and clinical chemistry could guide the veterinary surgeon towards the diagnosis of *T. equi* or *B. caballi* since horses showed a significant leukocytosis or anemia and hyperbilirubinemia, respectively; however PCR would be the test of choice in order to confirm the diagnosis. Information about the importance of a correct diagnosis of EP using a combination of techniques is essential in order to allow the early detection of cases and prevent the spread of the disease, as well as to avoid the common practice of treating horses without a laboratory diagnosis.

1. Introduction

Wild and domestic animals belonging to the *Equidae* family (horses, donkeys, mules and zebras) are affected by equine piroplasmosis, a tick-borne protozoan disease (Mehlhorn and Schein, 1998). Two intra-erythrocytic hemoprotozoans (*T. equi* and *B. caballi*) are the causal agents of this disease, which is found in most tropical, sub-tropical and temperate areas of the world and is transmitted by Ixodid ticks, including the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Dewaal, 1992; Homer et al., 2000). Mixed *T. equi* and *B. caballi* infections are known to happen when a common vector is present (Scoles and Ueti, 2015). Iatrogenic transmission by transfer of blood or through contaminated needles can also occur (Short et al., 2012). Both parasites may cause hyperacute, acute, subacute or chronic disease (Rothschild, 2013). Infected animals can recover although they may become carriers for several years (*B. caballi*) or life-long (*T. equi*), behaving as reservoirs for ticks (Knowles, 1996). EP is a disease causing major restrictions and economic losses in the international movement of horses (Friedhoff et al., 1990; Wise et al., 2013). Diagnosis of EP is difficult since the most common clinical findings (transient fever exceeding 40 °C, jaundice, petechial haemorrahages, hemoglobinuria, bilirubinuria and edema of the distal limbs) are variable and nonspecific in any of the clinical infections (peracute, acute, subacute and chronic) (Maurer, 1962). Abortions, inappetence, loss of body condition, poor performance and even death can also be observed in horses (Taylor et al., 1969; Zobba et al., 2008). Horses present hemolytic anemia as well as high bilirubin, and may show leukocytosis and hyperproteinemia (Kumar et al., 2002). Direct methods (peripheral blood smear examination, *in vitro* cultures

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and molecular tools) and indirect methods [complement fixation test (CFT), indirect fluorescent antibody test (IFAT) and competitive ELISA] have been developed to demonstrate infection or contact with the parasites (Garcia-Bocanegra et al., 2013; Holman et al., 1993).

Despite the information regarding hematological, molecular and serological approaches to the diagnosis of *T. equi* and *B. caballi* infections (Moretti et al., 2010; Ybanez et al., 2018), veterinary surgeons in endemic areas (such as Spain) often base the diagnosis and treatment of EP on clinical and epidemiological criteria, without having a confirmation of the presence of these parasites in the horse (Coultous et al., 2019; Leblond, 2019). The aims of this study were (1) to describe the presence of hematological and biochemical (total and direct bilirubin) changes in horses with clinical suspicion of EP, (2) to evaluate the comparative performance of different diagnostic tests in these horses, and (3) to present the most appropriate technique for the diagnosis of EP in these cases, which will result in a faster and more accurate resolution.

2. Materials and methods

A total of 140 horses of different breeds residing in different areas throughout Spain were included in the study. After a routine physical examination carried out by a veterinary surgeon, blood samples were collected from the jugular vein of horses that presented at least three of the most common clinical signs of EP: pyrexia (rectal temperature of 38.5 °C or higher), jaundice and pale mucous membranes. Blood samples were placed into two sterile tubes (with and without EDTA anticoagulant) and submitted on ice to the Equine Health Surveillance Unit (VISAVET Centre) between February 2016 and April 2019. For each horse included in the study, the tube without anticoagulant was centrifuged, and serum was separated and stored at -20 °C until biochemical and serological tests were carried out. The blood with EDTA was used for the hematological screening and the remaining blood was stored at -80 °C until DNA extraction.

2.1. Hematological and biochemical analysis

Hematological parameters [total red blood cell (RBC), packed cell volume (PCV), hemoglobin concentration (Hb), white blood cell (WBC) and platelet count] were determined by using an automatic counter system (Urit 2900 Vet Plus TS, URIT Medical Electronic Group Co, LTD, Guilin, China), whereas biochemical analyses (TB: total bilirubin, DB: direct bilirubin) were performed by liquid chemistry (DILABO, Ciudad Real, Spain) by using an automatic photometer (TECOM TC220, TECOM, Jiangxi, China) and the reference intervals for horses from a population of 120 healthy horses residing in Spain using the same reagents and photometer (data not published from the VISAVET Centre, Universidad Complutense de Madrid, Madrid, Spain). The RBC, PCV and Hb were used as indicators of anemia using the reference intervals from the automatic counter system.

2.2. Blood smear microscopic evaluation

Thin EDTA blood smears were prepared, stained with Diff Quick panoptic method and observed at 40x and under oil immersion to detect the presence of intra-erythrocytic piroplasms.

2.3. Competitive ELISA

Serum samples were thawed and used to detect equine IgG-specific antibodies against *T. equi* and *B. caballi* using two commercial cELISA kits (VMRD Inc., Pullman, WA, USA), which presented a sensitivity of 95.0% (*T. equi*) and 100% (*B. caballi*) and a specificity of 99.5% (*T. equi*) and 100% (*B. caballi*) (data derived from VMRD catalog 2017) (VMRD, 2017). Assays were performed according to the manufacturer´s instructions. Results were calculated from the optical densities measures at 620 nm and expressed as IP. Sera were classified as positive (IP ≥ 40%) or negative (IP < 40%).

2.4. DNA extraction

Total genomic DNA was extracted from 200 µl EDTA blood using the QIAamp DNA mini kit (Qiagen, Spain), according to the manufacturer´s protocol. Extracted DNA was preserved at −40 °C until multiplex real-time PCR was carried out.

2.5. Multiplex real-time PCR

A multiplex real-time PCR for the simultaneous detection of both hemoprotozoan parasites from horse blood samples was adapted from a previously published assay for the detection of *T. equi* (Kim et al., 2008) and *B. caballi* (Bhoora et al., 2010) using primers and TaqMan probes to specifically amplify the V4 hypervariable region of the 18S rRNA gene of *T. equi* and *B. caballi* and with a detection limit of 3.0×10^{-4} % PE (parasitized erythrocytes) for *T. equi* and 2.0×10^{-3}% PE for *B. caballi.*

The PCR mix contained a total reaction volume of 25 µl comprising 5x TaqMan QuantiFast Pathogen PCR master mix (Qiagen, Spain), 0.4 µM of each forward and reverse primer for both *B. caballi* and *T. equi* (Be_18SF, Be_18SR, Bc_18SF402 and Bc_18SR496), 0.2 µM of Be_18SP probe, 0.4 µM of Bc_18SP probe, and 5 µl of target DNA (Bhoora et al., 2010; Kim et al., 2008). The 5´-end of Be_18SP probe was labeled with FAM reporter dye and the 3´-end with BHQ1 quencher dye. Bc_18SP was labeled as follows: 5´-TexasRed, 3´-BHQ2. An internal DNA control template, plus an internal control primer/probe set (Qiagen, Spain) were also incorporated in the reaction. Positive controls (*T. equi* and *B. caballi* DNAs obtained in our laboratory from the blood of two horses with acute disease which tested positive to the PCR and were confirmed by sequencing the 18S rRNA gene) as well as a negative extraction control and a negative PCR control (ultrapure sterilized water) were included in the PCR assay.

Real-time PCR reactions were performed using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Cycling conditions used for either *T. equi* and *B. caballi* amplification were the following: initial denaturation cycle at 95 °C for 5 min, followed by 45 cycles of 1 s at 95 °C and 60 s at 55 °C. The collected fluorescence data was analyzed using the Bio-Rad CFX Manager (Version 3.1; Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Statistical analysis

Seropositivity rates by cELISA and PCR for both *T. equi* and *B. caballi* were analyzed using Chi square test (X^2). Furthermore, in order to determine the level of agreement between the different results obtained by molecular and serological tests for both parasites, Kappa coefficient (k) was calculated and assigned to the corresponding ranges (Landis and Koch, 1977) (Supplementary data).

The statistical association between the different variables was analyzed using Mann-Whitney and Kruskal-Wallis tests for continuous numerical variables with a non-normal distribution and by t-Student and ANOVA tests for those whose distribution was normal. Kolmogorov-Smirnov test was used to check the normality of the variables. The analyses were performed in the following combinations: (A) PCR result (positive/negative) vs cELISA IP; (B) PCR result (positive/negative) vs hematological and biochemical quantitative values; and (C) cELISA result (positive/negative) vs hematological and biochemical results. Besides, the strength and direction of the association between IP results and hematological and biochemical values was measured by calculating the Pearson (r) and Spearman's correlation coefficient (p) for normally and non-normally distributed variables, respectively. All evaluations were conducted for both parasites using the software IBM SPSS Statistics version 20. Differences were considered to be statistically significant with a p value < 0.05.

3. Results

The horses included in the survey showed the following mean values for hematological and biochemical parameters: RBC ($6.7 \pm 1.4 \times 10^6$/μL), Hb concentration (10.9 ± 2.2 g/dL), PCV ($31.0 \pm 6.4\%$), WBC ($8.5 \pm 2.9 \times 10^3$/μL), platelet count ($129 \pm 60 \times 10^3$/μL), TB (2.7 ± 2.2 mg/dL) and DB (0.34 ± 0.44 mg/dL). Of the 140 horses, nineteen (13.6%), sixty-eight (48.6%) and sixty-four (45.7%) had decreased values of RBC, Hb and PCV, respectively. Furthermore, an increase in the value of TB and DB was present in 35.0% (49/140) and 4.3% (6/140) horses, respectively.

The examination of thin blood smears revealed one or two basophilic pear-shaped intraerythrocyte parasites with a length of 2–5 μm (*B. caballi*) or smaller merozoites with a pyriform, round or ovoid shape, some of which were forming the "maltese cross" (*T. equi*) (Malekifard et al., 2014) in only 9.3% (13/140) of the studied horses.

Competitive ELISA tests revealed that 71 of the 140 horses (50.7%) included in the survey showed antibodies to EP. The seropositivity rates for *T. equi* and *B. caballi* in these animals were 44.3% (62/140) and 16.4% (23/140), respectively. Furthermore, 14 (10.0%) horses tested seropositive for both piroplasmids. Multiplex real-time PCR based on the 18S rRNA gene showed that 60 of the 140 animals sampled (42.9%) were positive for EP. Of these, 55 horses (39.3%) were positive for *T. equi* and 8 (5.7%) for *B. caballi*. Co-infection with both hemoparasites was found in only 3 (2.1%) horses. Statistical analysis of these data by X^2 test demonstrated that the percentage of qPCR-positives and the seropositives for *T. equi* were significantly higher (p value < 0.001) than for *B. caballi*. A statistically significant difference (p value < 0.001) in the diagnosis of *B. caballi* infections between PCR and cELISA was showed. However, no significant differences (p value = 0.265) were observed in the case of *T. equi*.

Overall, concordance value between the molecular and serological tests for the diagnostic of EP showed a moderate strength of agreement (kappa value = 0.587) (Landis and Koch, 1977). Kappa statistic between both diagnostic techniques (PCR and cELISA) was higher for the detection of *T. equi* infections ($k = 0.575$) than for *B. caballi* ($k = 0.401$), being classified in the range of moderate and fair strength of association, respectively (Landis and Koch, 1977).

Results revealed the existence of a statistically significant association between PCR results and cELISA IP values for both *T. equi* (p value < 0.001) and *B. caballi* (p value = 0.001) by using the Mann-Whitney U test. Real-time PCR positive horses showed a significantly higher cELISA IP (Fig. 1).

Regarding the comparison between the positive/negative EP PCR and positive/negative EP cELISA results with hematological and biochemical values, statistically significant differences were found for several parameters; RBC (p value = 0.020) and Hb (p value = 0.042) were significantly lower in EP PCR positive horses, while WBC were significantly higher in EP positive horses, using both PCR (p value = 0.002) and cELISA (p value = 0.001) (Table 1).

Horses with a positive PCR for *T. equi* presented significantly higher WBC counts than horses with a negative PCR result (p value = 0.001). This finding was also obtained when using the cELISA (p value = 0.001) (Table 2). Horses with a positive PCR for *B. caballi* had significantly lower values of RBC (p value = 0.004), Hb (p value < 0.001), PCV (p value = 0.001) and platelet count (p value < 0.001). When using the *B. caballi* cELISA test, the same significant associations were also revealed for RBC (p value < 0.001), Hb (p value = 0.001), PCV (p value = 0.007) and platelet count (p value < 0.001) (Table 3). The differences were not significant for any of the serum biochemical parameters (TB and DB) between positive and negative animals to *T. equi* and *B. caballi* when using PCR or cELISA.

Statistical analysis (Pearson and Spearman coefficients) carried out to evaluate the relationship between the hematological/biochemical values and cELISA IP showed that, for *T. equi*, there was a statistically significant positive correlation with WBC count ($p = 0.239$; p value = 0.004). In the case of *B. caballi*, a statistically significant negative correlation was showed with the following hematological parameters: RBC ($r = -0.383$, p value < 0.001), Hb ($p = -0.264$, p value = 0.002), PCV ($p = -0.293$, p value < 0.001) and platelet count ($p = -0.226$, p value = 0.007); a milder proportional direct correlation was observed with the total bilirubin value ($p = 0.138$, p value = 0.104).

4. Discussion

This study presents the serological, molecular, hematological and biochemical results obtained from 140 horses with a clinical suspicion of EP residing in Spain. The clinical signs shown by all these horses (pyrexia, pale mucous membranes and icterus) have been described in other studies for horses with acute EP (Hailat et al., 1997; Ionita et al., 2018). Our study reported that only 42.9% of horses with clinical signs suggestive of EP were actually positive by PCR, suggesting the need of a confirmatory diagnosis in these horses before EP treatment, since these clinical signs are not pathognomonic of the disease (Rothschild, 2013).

Blood smears examination revealed the presence of intraerythrocyte parasites compatible with *T. equi* and/or *B. caballi* in only 13 of the 140 horses, even when 60 horses tested positive for *T. equi*, *B. caballi* or both; showing that even though diagnosis by microscopic examination is easy to perform and most useful during the acute phase of infection, false-negative results are common since the number of parasites in blood may be low (mainly in *B. caballi* parasitemia) even during severe

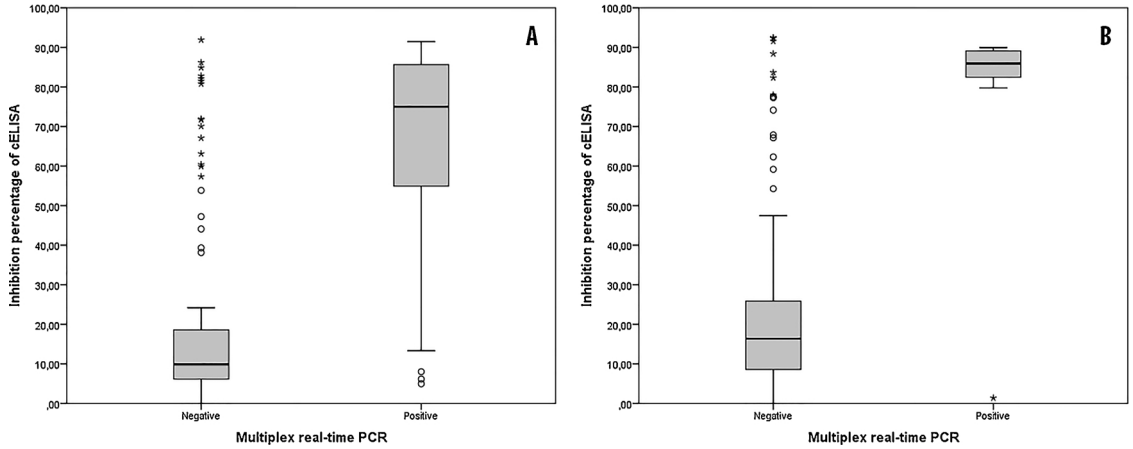

Fig. 1. The graphs describe the relationship between positive/negative PCR results and cELISA IP values for *T. equi* (Graph A) and for *B. caballi* (Graph B). Outliers represent either PCR negative horses with close (○)/distant (*) IP values from 40%, or PCR positive horses with a very low cELISA IP.

Table 1
Hematological and biochemical values from 140 horses with clinical signs suggestive of EP. Horses have been divided by their EP result (positive or negative) by PCR and cELISA. Values are shown as means (± standard deviation) for each parameter.

Parameter (unit)	PCR			cELISA			Reference interval[a]
	Positive (n = 60)	Negative (n = 80)	p-value	Positive (n = 71)	Negative (n = 69)	p-value	
Red blood cell (/10^6µl)	6.4 (± 1.7)	7.0 (± 1.1)	0.020[b,*]	6.6 (± 1.4)	6.9 (± 1.4)	0.206[b]	5.3 – 13.0
Hemoglobin (g/dl)	10.3 (± 2.5)	11.3 (± 1.8)	0.042[c,*]	10.7 (± 2.1)	11.1 (± 2.2)	0.422[c]	10.8 – 18.8
Packed cell volumen (%)	29.4 (± 7.3)	32.2 (± 5.3)	0.060[c]	30.6 (± 6.1)	31.4 (± 6.6)	0.654[c]	30.0 – 53.0
White blood cell (/10^3 mm^3)	9.3 (± 2.7)	8.0 (± 2.9)	0.002[c,*]	9.4 (± 3.0)	7.7 (± 2.5)	0.001[c,*]	5.0 – 11.0
Platelet count (/10^3 mm^3)	120 (± 61)	135 (± 59)	0.142[c]	125 (± 53)	132 (± 66)	0.623[c]	96 - 360
Total bilirubin (mg/dl)	2.9 (± 2.6)	2.5 (± 1.8)	0.616[c]	2.4 (± 1.4)	3.0 (± 2.8)	0.209[c]	0.1 – 2.5
Direct bilirubin (mg/dl)	0.40 (± 0.65)	0.29 (± 0.15)	0.159[c]	0.29 (± 0.14)	0.38 (± 0.61)	0.415[c]	0.01 – 0.67

[a] Reference intervals for haematology were determined from a Urit 2900 Vet Plus TS (URIT Medical Electronic Group Co, LTD, Guilin, China), and from a reference population of 120 healthy horses residing in Spain using the same reagents (DILABO, Ciudad Real, Spain) and photometer (TECOM TC220, TECOM, Jiangxi, China) for biochemistry (data not published from the Equine Health Surveillance Unit, VISAVET Centre, Madrid, Spain).
[b] By t-Student test.
[c] By Mann-Whitney U test.
* Represents statistical significance for $p < 0.05$.

Table 2
Comparison of hematological and biochemical parameters between *T. equi*-positive and *T. equi*-negative horses found by real-time PCR or by cELISA. Values are shown as means (± standard deviation) for each parameter.

Parameter (unit)	PCR			cELISA			Reference interval[a]
	Positive (n = 55)	Negative (n = 85)	p-value	Positive (n = 62)	Negative (n = 78)	p-value	
Red blood cell (/10^6µl)	6.6 (± 1.5)	6.8 (± 1.3)	0.494[b]	6.8 (± 1.3)	6.7 (± 1.5)	0.510[b]	5.3 – 13.0
Hemoglobin (g/dl)	10.6 (± 2.2)	11.0 (± 2.1)	0.508[c]	11.0 (± 1.7)	10.7 (± 2.4)	0.425[c]	10.8 – 18.8
Packed cell volumen (%)	30.4 (± 6.6)	31.4 (± 6.2)	0.594[c]	31.7 (± 5.0)	30.5 (± 7.3)	0.268[c]	30.0 – 53.0
White blood cell (/10^3 mm^3)	9.5 (± 2.7)	7.9 (± 2.9)	0.001[c*]	9.5 (± 3.1)	7.7 (± 2.5)	0.001[c,*]	5.0 – 11.0
Platelet count (/10^3 mm^3)	126 (± 59)	130 (± 61)	0.821[c]	132 (± 52)	126 (± 66)	0.264[c]	96 - 360
Total bilirubin (mg/dl)	2.9 (± 2.7)	2.5 (± 1.8)	0.968[c]	2.4 (± 1.4)	2.9 (± 2.7)	0.185[c]	0.1 – 2.5
Direct bilirubin (mg/dl)	0.42 (± 0.68)	0.28 (± 0.15)	0.070[c]	0.29 (± 0.15)	0.37 (± 0.58)	0.347[c]	0.01 – 0.67

[a] Reference intervals for haematology were determined from a Urit 2900 Vet Plus TS (URIT Medical Electronic Group Co, LTD, Guilin, China), and from a reference population of 120 healthy horses residing in Spain using the same reagents (DILABO, Ciudad Real, Spain) and photometer (TECOM TC220, TECOM, Jiangxi, China) for biochemistry (data not published from the Equine Health Surveillance Unit, VISAVET Centre, Madrid, Spain).
[b] By t-Student test.
[c] By Mann-Whitney U test.
* Represents statistical significance for $p < 0.05$.

Table 3
Comparison of hematological and biochemical parameters between *B. caballi*-positive and *B. caballi*-negative horses found by real-time PCR or by cELISA. Values are shown as means (± standard deviation) for each parameter.

Parameter (unit)	PCR			cELISA			Reference interval[a]
	Positive (n = 8)	Negative (n = 132)	p-value	Positive (n = 23)	Negative (n = 117)	p-value	
Red blood cell (/10^6µl)	4.7 (± 1.5)	6.8 (± 1.3)	0.004[b,*]	5.6 (± 1.4)	6.9 (± 1.3)	< 0.001[b,*]	5.3 – 13.0
Hemoglobin (g/dl)	7.6 (± 2.4)	11.1 (± 2.0)	< 0.001[c,*]	9.3 (± 2.3)	11.2 (± 2.0)	0.001[c*]	10.8 – 18.8
Packed cell volumen (%)	22.3 (± 7.2)	31.5 (± 6.0)	0.001[c,*]	27.4 (± 7.2)	31.7 (± 6.0)	0.007[c,*]	30.0 – 53.0
White blood cell (/10^3 mm^3)	8.7 (± 2.5)	8.5 (± 2.9)	0.723[c]	8.5 (± 2.2)	8.5 (± 3.0)	0.710[c]	5.0 – 11.0
Platelet count (/10^3 mm^3)	64 (± 32)	132 (± 59)	< 0.001[c,*]	85 (± 30)	137 (± 61)	< 0.001[c,*]	96 - 360
Total bilirubin (mg/dl)	2.6 (± 1.6)	2.7 (± 2.2)	0.950[c]	2.3 (± 1.2)	2.7 (± 2.4)	0.679[c]	0.1 – 2.5
Direct bilirubin (mg/dl)	0.23 (± 0.06)	0.34 (± 0.46)	0.091[c]	0.27 (± 0.06)	0.35 (± 0.48)	0.783[c]	0.01 – 0.67

[a] Reference intervals for haematology were determined from a Urit 2900 Vet Plus TS (URIT Medical Electronic Group Co, LTD, Guilin, China), and from a reference population of 120 healthy horses residing in Spain using the same reagents (DILABO, Ciudad Real, Spain) and photometer (TECOM TC220, TECOM, Jiangxi, China) for biochemistry (data not published from the Equine Health Surveillance Unit, VISAVET Centre, Madrid, Spain).
[b] By t-Student test.
[c] By Mann-Whitney U test.
* Represents statistical significance for $p < 0.05$.

infection (Wise et al., 2014).

The percentage of seropositivity for *T. equi* (44.3%) and *B. caballi* (16.4%) found in horses presenting clinical signs of EP of this study was obviously higher than that reported for a general population of healthy horses in central Spain (Camino et al., 2018). Our findings regarding the percentage of *T. equi* and *B. caballi* positives by PCR in the horses with clinical suspicion were in agreement with the studies carried out in most endemic regions where, in general, *T. equi* infected horses are

more common than those infected by *B. caballi* (Bartolome Del Pino et al., 2016; Posada-Guzman et al., 2015); however this is not the case in other regions such as Mongolia (Munkhjargal et al., 2013).

Compared to the results for *T. equi*, where the number of positive horses detected by cELISA and real-time PCR was very similar, a slightly lower agreement was seen between both tests for *B. caballi*, since a significantly higher number of horses tested positive by cELISA than by real-time PCR. While positive results by PCR indicate current active parasite infestation, cELISA positive results show past or chronic infections (Butler et al., 2012). According to this, our results could involve a lower presence of *B. caballi* carrier horses than *T. equi* in our study. This would be in agreement with the fact that the carrier state in horses infected with *B. caballi* is short and the parasite usually disappears from the bloodstream after treatment or 1-4 years post-infection in natural recovery, while *T. equi* is more difficult to eliminate due to the parasitemia being typically higher, so the horses can remain as life-long carriers (Bruning, 1996). Nevertheless, the differences in the agreement for both parasites could also be due to the fact that these two techniques have different targets [PCR targets the 18S gene while *T. equi* cELISA targets EMA-1 (equi merozoite antigen 1) and *B. caballi* cELISA targets RAP-1 (rhoptry-associated protein 1)] (Bhoora et al., 2010; Kappmeyer et al., 1999; Katz et al., 2000; Kim et al., 2008; Knowles et al., 1992).

This is the first study to evaluate the association between PCR results and cELISA IP values. In the horses with clinical signs suggestive of EP included in our study, a high IP in the *B. caballi* cELISA was associated with positive PCR results, whereas for *T. equi*, a low IP was also associated with positive PCR results. This fact could be related to the kinetics of antibody responses; studies in experimentally infected horses have demonstrated the presence of *T. equi* in bloodstream by PCR from the first week post-inoculation, whereas initial detection of infection by cELISA was possible between 5 and 12 weeks (Grause et al., 2013). Regarding *B. caballi*, Schwint et al. (2009) were able to demonstrate the presence of the parasite and the antibodies by means of both PCR and cELISA at the same time [within 8 (inoculation) or 12 (tick borne) days post-transmission].

According to our results, hematological alterations caused by EP included a decrease in the RBC count and Hb concentration, with increased values of WBC. Similar findings were reported in EP positive equids in Egypt, although no significant differences were found regarding WBC (Mahmoud et al., 2016). There were differences in the blood count between *T. equi/B. caballi*-parasitized horses. Horses positive for *T. equi* (by PCR and/or cELISA) showed a significant rise in WBC, whereas horses positive for *B. caballi* presented a significant decrease of RBC, PCV, Hb and platelet counts. Even though leukopenia is more frequent in acute forms of EP (Dewaal, 1992), leukocytosis in *T. equi* infections was also seen in a study carried out in racehorses in Jordan (Hailat et al., 1997) and it may be related with its ability to invade the peripheral blood mononuclear cells at the initial stage (Mehlhorn and Schein, 1998). In general, *T. equi* presence has been associated with a more severe disease (Camacho et al., 2005; Zobba et al., 2008) but, unexpectedly, in this study a significantly more severe anemia was observed in *B. caballi* infected horses by using both cELISA (RBC p value < 0.001; Hb p value $= 0.002$; PCV p value $= 0.002$) and PCR (RBC p value $= 0.001$; Hb p value $= 0.002$; PCV p value $= 0.003$), and thrombocytopenia was also observed in these horses (p value < 0.001). Thrombocytopenia is a very characteristic feature of canine babesiosis (Mierzejewska et al., 2014), and it has also been reported in studies in equine (Camacho et al., 2005; Zobba et al., 2008), although normal levels have been found elsewhere (Ionita et al., 2018).

Finally, a positive correlation was found which suggests that, as the cELISA IP increases, the levels of total bilirubin and number of WBC also raise, for *B. caballi* and *T. equi*, respectively. The increase in the IP was also associated with a significant decrease of RBC, PCV, Hb and platelet count in *B. caballi* infections. We did not observe a significant difference with the direct bilirubin values; which may be explained by the fact that hyperbilirubinemia, a consequence of hemolytic anemia, is the most frequent biochemical alteration in EP and it is produced by an increase in the indirect (unconjugated) bilirubin (Rothschild, 2013).

5. Conclusions

Data presented here confirmed that *T. equi* infections are more prevalent than *B. caballi* infections in horses with clinical signs suggesting EP in Spain; however, only 80 (57%) out of 140 horses with clinical signs compatible with EP were actually cELISA and/or PCR positive. We believe that most positive horses in our study could be *T. equi*-carriers suffering a relapse (acute or sub-acute disease), while *B. caballi* would be mainly responsible for primary infections (acute or sub-acute disease). When using cELISA in horses with clinical signs of EP, a high IP ($> 78\%$) for *B. caballi* would be suggestive of an active infection, although PCR would be recommended for confirmation. The haemogram could guide the veterinary surgeon towards the diagnosis of *T. equi* or *B. caballi*; however PCR would be the test of choice in order to confirm the diagnosis. Information about the importance of correct diagnosis, treatment and control measures of EP should be given to the veterinarians in order to allow the early detection of cases and prevent the spread of the disease, as well as to avoid the common practice of treating horses without a laboratory diagnosis of EP.

Animal welfare

The whole sampling related to this study underwent the Ethical Committee of the Universidad Complutense de Madrid. The owners of the horses included in the study received an information sheet with all the information regarding the study and they signed an informed consent that would allow the samples to be used in further studies.

Declaration of Competing Interest

The authors declare no conflict of interest in the subject, matter or materials discussed in this manuscript.

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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.vetpar.2019.108928.

References

Bartolome Del Pino, L.E., Nardini, R., Veneziano, V., Iacoponi, F., Cersini, A., Autorino, G.L., Buono, F., Scicluna, M., 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.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., Van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2010. Development and evaluation of 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.

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

Butler, C.M., Sloet van Oldruitenborgh-Oosterbaan, M.M., Stout, T.A., van der Kolk, J.H., Wollenberg, L., Nielen, M., Jongejan, F., Werners, A.H., Houwers, D.J., 2012. Prevalence of the causative agents of equine piroplasmosis in the South West of The Netherlands and the identification of two autochthonous clinical Theileria equi

infections. Vet. J. 193, 381–385.
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.
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.
Coultous, R.M., Phipps, P., Dalley, C., Lewis, J., Hammond, T.A., Shiels, B.R., Weir, W., Sutton, D.G.M., 2019. Equine piroplasmosis status in the UK: an assessment of laboratory diagnostic submissions and techniques. Vet. Rec. 184, 95.
Dewaal, D.T., 1992. Equine piroplasmosis - a review. Br. Vet. J. 148, 6–14.
Friedhoff, K.T., Tenter, A.M., Muller, I., 1990. Haemoparasites of equines: impact on international trade of horses. Rev. Sci. Technol. 9, 1187–1194.
Garcia-Bocanegra, I., Arenas-Montes, A., Hernandez, E., Adaszek, L., Carbonero, A., Almeria, S., Jaen-Tellez, J.A., Gutierrez-Palomino, P., Arenas, A., 2013. Seroprevalence and risk factors associated with Babesia caballi and Theileria equi infection in equids. Vet. J. 195, 172–178.
Grause, J.F., Ueti, M.W., Nelson, J.T., Knowles, D.P., Kappmeyer, L.S., Bunn, T.O., 2013. Efficacy of imidocarb dipropionate in eliminating Theileria equi from experimentally infected horses. Vet. J. 196, 541–546.
Hailat, N.Q., Lafi, S.Q., al-Darraji, A.M., al-Ani, F.K., 1997. Equine babesiosis associated with strenuous exercise: clinical and pathological studies in Jordan. Vet. Parasitol. 69, 1–8.
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.
Homer, M.J., Aguilar-Delfin, I., Telford 3rd, S.R., Krause, P.J., Persing, D.H., 2000. Babesiosis. Clin. Microbiol. Rev. 13, 451–469.
Ionita, M., Nicorescu, I.M., Pfister, K., Mitrea, I.L., 2018. Parasitological and molecular diagnostic of a clinical Babesia caballi outbreak in Southern Romania. Parasitol. Res. 117, 2333–2339.
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, 2285–2290.
Katz, J., Dewald, R., Nicholson, J., 2000. Procedurally similar competitive immunoassay systems for the serodiagnosis of Babesia equi, Babesia caballi, Trypanosoma equiperdum, and Burkholderia mallei infection in horses. J. Vet. Diagn. Invest. 12, 46–50.
Kim, C.M., Blanco, 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.
Knowles Jr., D., 1996. Equine babesiosis (piroplasmosis): a problem in the international movement of horses. Br. Vet. J. 152, 123–126.
Knowles Jr., 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. 30, 3122–3126.
Kumar, S., Malhotra, D.V., Nichani, A.K., 2002. Identification of immunoreactive polypeptides of Babesia equi parasite during immunization. Vet. Parasitol. 107, 295–301.
Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33, 159–174.
Leblond, A., 2019. Equine piroplasmosis - the view of a practitioner from an endemic region. Vet. Rec. 184, 92–94.
Mahmoud, M.S., 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 hematological approaches. Parasit. Vectors 9, 260.
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.
Maurer, F.D., 1962. Equine piroplasmosis-another emerging disease. J. Am. Vet. Med. Assoc. 141 699-&.
Mehlhorn, H., Schein, E., 1998. Redescription of Babesia equi Laveran, 1901 as Theileria equi Mehlhorn, Schein 1998. Parasitol. Res. 84, 467–475.
Mierzejewska, E.J., Welc-Faleciak, R., Bednarska, M., Rodo, A., Bajer, A., 2014. The first evidence for vertical transmission of Babesia canis in a litter of Central Asian Shepherd dogs. Ann. Agric. Environ. Med. 21, 500–503.
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.
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.
Posada-Guzman, M.F., Dolz, G., Romero-Zuniga, J.J., Jimenez-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., 236278 2015.
Rothschild, C.M., 2013. Equine piroplasmosis. J. Equine Vet. Sci. 33, 497–508.
Schwint, O.N., Ueti, M.W., Palmer, G.H., Kappmeyer, L.S., Hines, M.T., Cordes, R.T., Knowles, D.P., Scoles, G.A., 2009. Imidocarb Dipropionate Clears Persistent Babesia caballi infection with elimination of transmission potential. Antimicrob. Agents Chemother. 53, 4327–4332.
Scoles, G.A., Ueti, M.W., 2015. Vector ecology of equine piroplasmosis. Annu. Rev. Entomol. 60, 561–580.
Short, M.A., Clark, C.K., Harvey, J.W., Wenzlow, N., Hawkins, I.K., Allred, D.R., Knowles, D.P., Corn, J.L., Grause, J.F., Hennager, S.G., Kitchen, D.L., Traub-Dargatz, J.L., 2012. Outbreak of equine piroplasmosis in Florida. J. Am. Vet. Med. Assoc. 240, 588–595.
Taylor, W.M., Bryant, J.E., Anderson, J.B., Willers, K.H., 1969. Equine piroplasmosis in the United States–a review. J. Am. Vet. Med. Assoc. 155, 915–919.
VMRD, 2017. Equine piroplasmosis. Veterinary Diagnostic Test Kits and Reagents. pp. 3–4.
Wise, L.N., Kappmeyer, L.S., Mealey, R.H., Knowles, D.P., 2013. Review of equine piroplasmosis. J. Vet. Intern. Med. 27, 1334–1346.
Wise, L.N., Pelzel-McCluskey, A.M., Mealey, R.H., Knowles, D.P., 2014. Equine piroplasmosis. Vet. Clin. North Am. Equine Pract. 30, 677–693.
Ybanez, A.P., Ybanez, R.H.D., Talle, M.G., Arreglo, R.M.T., Geens, M.J.C., Villas 3rd, J.G.I., Villar, S.R., Laruga, C.L., Cao, S., Moumouni, F.P.A., Liu, M., Igarashi, I., Xuan, X., 2018. Serological and molecular detection of Theileria equi and Babesia caballi in Philippine horses. Ticks Tick. Dis. 9, 1125–1128.
Zobba, R., Ardu, M., Niccolini, S., Chessa, B., Manna, L., Cocco, R., Parpaglia, M.L.P., 2008. Clinical and laboratory findings in equine piroplasmosis. J. Equine Vet. Sci. 28, 301–308.