Risk factors and haemato-biochemical parameters associated with *Theileria equi* infection in Central Italy

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Research Article

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Abstract

Background: Equine piroplasmosis is a tick-borne disease affecting equids caused by protozoa *Theileria equi* and *Babesia caballi*, causing losses in the horse industry for impaired performance and constraint to the international movement. In endemic areas, clinical diagnosis is difficult because the most frequent form is the asymptomatic carrier status and even in symptomatic cases, signs are not pathognomonic. Laboratory analyses are required to confirm the diagnosis and assess therapy.

Methods: A total of 438 horse and donkey samples were collected by practitioners during their routine activities and for clinical suspicion of piroplasmosis. The anamnestic information as signs, individual data and management practises were collected by a standardized form. The samples were screened for piroplasmosis and other infectious diseases in differential diagnosis and haemato-biochemical parameters, and thus assigned to three groups: confirmed cases, unconfirmed suspects and controls. Univariate logistic regressions were performed using groups as outcome and anamnestic data as independent variables. Differences in haemato-biochemical parameters among groups were evaluated by Kruskal-Wallis H-test, followed, when significant ($P < 0,05$), by Bonferroni correction for multiple comparisons.

Results: Nineteen confirmed *T. equi* cases and one *B. caballi* were detected, thus the study was focused only on the former parasite**.** Among anamnestic variables, only age (> 6 years) increases the probability of infection compared to the controls, while ectoparasiticide treatments seem to have a protective effect. A decrease in platelet count was the only haemato-biochemical parameter associated with *T. equi* infection.

Comparing with healthy subjects, total and direct bilirubin, monocytes, basophils, MCH and gamma protein fraction showed higher values whereas CK, eosinophils, RBC, HGB, HCT, RDW, albumin and A/G ratio showed lower values, despite being mostly within physiological range. These findings resulted common to *T. equi* and other affections with similar signs.

Conclusions: This study confirms the diagnosis of *T. equi* infection as an issue, for similarities regarding anamnesis and laboratory findings with other diseases; but still providing the practitioners with data useful for the laboratory results interpretation. Moreover, it focuses the attention on prevention measures, as external antiparasitic treatments and good management practices that, with regular laboratory checks, can reduce the spread of the disease.

Background

Equine piroplasmosis (EP) is a tick-borne disease of equids caused by two species of intra-erythrocytic protozoa named *Theileria equi* and *Babesia caballi.* EP is responsible for significant sanitary and economical losses in the horse industry due to impaired performance and also to the constraint to the international movement [1, 2]. EP distribution is correlate to that of its vectors, ticks of genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* [3], being thus endemic in tropical and temperate areas, including Central-Southern regions of Italy [4].

The clinical diagnosis of EP in such areas presents several issues and therefore can be arduous. Although peracute, acute and chronic forms are reported, the most frequent manifestation of the infection by both parasites is the unapparent carrier status with non-pathognomonic signs of disease [5, 6]. Even in symptomatic cases, the signs do not allow a differential diagnosis between the two parasites which is crucial for the therapy setting, therefore the laboratory diagnosis is necessary to detect haemato-biochemical alterations and/or presence of parasites.

Methods employed are usually represented by complete blood cells count (CBC), clinical biochemistry, stained blood smears, serological tests, such as indirect fluorescent antibody test (IFAT), ELISA, andreal-time PCR methods [7], using specific protocols, as described and recommended by World Animal Health Organization (OIE)[a]. The reliability of these methods and the results interpretation could be affected or complicated by intrinsic characteristics of the methods, i.e. lack of sensitivity during chronic infections or in the presence of particular parasite strains (ELISA), cross-reactivity (IFAT); or to the host-parasite interaction, i.e. antibody persistence and carrier status with segregation of the parasite in capillaries and bone marrow [6].

Within this diagnostic framework, the choice of treating a positive subject must be carefully considered, especially for animals having an economic or personal worth. In fact, severe side effects such as hepatotoxicity and nephrotoxicity are reported using imidocarb in the *T. equi* sterilization protocol, that requires a dose of 340 mg/100 kg body weight, four times with an inter-dose interval of 72 h [8, 9, 10].

The aim of this study was to investigate possible risks factors, comparing the haemato-biochemical results in animals with acute EP infection with healthy horses (controls) and with those with clinical signs compatible with the presence of EP, but not confirmed as infected; in order to identify the practices and the parameters useful respectively to the owners to prevent the disease and to the practitioners for the diagnosis and for the assessment of therapy protocols. Due to the laboratory results, that detected only one *B. caballi* case, the present paper focused on *T. equi*.

Methods

Sampling and anamnestic form

The sampling was carried out on the equine population of Lazio and Tuscany, regions, Central Italy, between May 2013 and November 2014. Sixty practitioners joined the project and recruited the animals (suspect cases and controls) during their routine activities. The eligibility criteria for the suspect case for piroplasmosis were initially: temperature > 39°C and at least one sign among anaemia, haematuria, petechial haemorrhages and jaundice, observed during the clinical examination. Controls were selected either in farms with similar characteristics to those where the suspected cases came from or by the contemporary sampling of two asymptomatic subjects together with the suspect case within the same premise. Since the expected number of cases could not be forecast, a ratio of 10:1 between controls and suspect cases has been pre-set, being this ratio assuring the highest statistical power to the study [11].

Blood samples were obtained by jugular venipuncture from each animal, collected in tubes with and without EDTA, and kept refrigerated until delivered to laboratories where sera were separated by centrifugation and frozen until assayed. Contextually to the sampling, an anamnestic data form was administered to the owners, who gave prior consent for the procedure. Information requested by the form are listed in Table 1.

Table 1
Information requested by the anamnestic data collection form administrated to the owners and available choices.

Variable	Available choices
Individual anamnesis	
Species	Horse, Mule, Donkey
Gender	Male, Gelding, Female
Age	Year of birth
Fur colour	Light, Dark
Breed	Autochthonous[a], Saddler, Trotter, Galloper, Other
Attitude	Meat production, Equestrian/Horse show, Work, Reproduction
Presence of ticks (at the sampling)	No, Yes
Recent Introduction	No, Yes
Management practices	
Housing	Inner, Access to outer
	Single, Group
Vaccination	No, Yes
External parasiticide treatment	No, Yes
Deworming	No, Yes
Clinical signs	Anaemia, Ataxia, Abdominal cramping, Fever, Haematuria, Jaundice, Lack of appetite, Lethargy, Lymphadenomegaly, Oedema, Petechial haemorrhages, Tachycardia, Tachypnea, Weakness, Weight loss
[a]Italian autochthonous breeds: Avelignese; Bardigiano; Cavallo Agricolo Italiano; Lipizzano; Maremmano; Murgese; Pony Esperia; Salernitano; Siciliano; Tolfetano	

Three levels of potential bias were considered: selection, recall and interviewer bias. Since practitioners were involved in the recruitment of suspect cases, a selection bias could have occurred, being some categories of horses (equestrian and sportive) kept more under veterinary control than those bred for meat production. In order to minimize this bias all practitioners that had ever submitted an equine sample to our laboratory in the previous years were invited to a presentation of the study, explaining aims and protocols and during which the collaboration in sampling all categories of equids was requested. To reduce possibility of collecting different anamnestic data between case and controls (recall bias) and minimize interviewer interferences (interviewer bias), the anamnestic form was designed in a structured scheme.

Laboratory analyses

A panel of serological, biomolecular and haemato-biochemical tests was performed on all samples (Table 2). Leptospirosis, anaplasmosis and equine infectious anaemia are included in the differential laboratory diagnosis in case of jaundice and anaemia. In order to exclude these diseases, the following tests were performed: micro agglutination test andreal-time PCR against leptospirosis [12–13]; IFAT (*A. phagocytophilum* IFA IgG Antibody Kit; Fuller Laboratories®, CA, USA) and nested PCR [14] for anaplasmosis, and ELISA for equine infectious anaemia [15].

Table 2

Serological and haemato-biochemical analyses performed on the samples collected during the study. Reference intervals were set as reported by Blood et al., 1994 [40], Aguggini et al., 2006 [41] and Riond et al. (2009) [42]. Value within round brackets are the reference values for percentage of WBC components.

Theileria equi	***Babesia equi* Antibody Test Kit, cElisa VMRD®**			
	Real time PCR (Kim et al. 2009) [17]			
Babesia caballi	*Babesia caballi* Antibody Test Kit, cELISA VMRD®			
	Real time PCR (Bhoora et al. 2010) [16]			
Leptospira spp.	Microscopic Agglutination Test			
	Real time PCR (QIAGEN®)			
Equine Infectious Anaemia	CTB-ELISA (Nardini et al. 2016) [15]			
Anaplasma phagocitophilum	Indirect Immunofluorescent Test			
	Nested PCR (Massung et al., 1998) [14]			
Complete blood count (10^3/µL)	WBC	6.00–10.0	HGB	11.0–15.0
	Neutrophils	2.70–7.60 (47–76%)	HCT	33.0–42.0
	Lymphocytes	1.50–4.50 (25–45%)	MCV	37.0–59.0
	Monocytes	0.00–0.80 (0–8%)	MCH	13.0–20.0
	Eosinophils	0.00–0.40 (0–4%)	MCHC	31.3–39.0
	Basophils	0.00–0.1 (0–1%)	RDW	0.0–27.0
	RBC	6.00–9.90	PLT	100–350
Blood Protein Electrophoresis	Albumin	2.10–3.00 g/dL	Beta 2	0.40–0.70 g/dL
	Alpha 1	0.70–1.20 g/dL	Gamma	1.70–2.50 g/dL
	Beta 1	0.30–0.70 g/dL	A/G ratio	0.93–1.65
Biochemical analyses	Total protein		5.50–7.70 g/dL	
	AST		200–400 U/L	
	CK		< 500 U/L	
	Total bilirubine		0.20–6.00 mg/dL	
	Direct bilirubine		< 0.40 mg/dL	

Theileria equi	*Babesia equi* Antibody Test Kit, cElisa VMRD®
	Real time PCR (Kim et al. 2009) [17]

WBC: total white blood cells, RBC: total red blood cells count, HGB: haemoglobin level, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, RDW: red cell distribution width, PLT: platelets, TP: total protein, AST: aspartate aminotransferase, CK: creatinphosphokinase.

Two c-ELISA assays were performed according to the manufacturer`s instructions: *B. caballi* Antibody test kit (VMRD®, Inc, WA, USA) uses a recombinant rhoptry-associated protein 1 (rap-1) as antigen, while *B. equi* Antibody test kit (VMRD®, Inc, WA, USA) employs a recombinant Equi Merozoite Antigen 1 (ema-1).

Two in house real-time PCRs were employed on all samples. The one for *B. caballi* amplifies a 95-base-pair (bp) fragment in the V4 hyper variable region of 18S rRNA gene of *B. caballi* with primers F: Bc-18SF402; R and Bc-18SR496 reported by Bhoora et al. [16], used at a concentration of 0.9 μM and TaqMan 5120 MGB TM probe (FAM MGB, Bc-18SP) employed at 0.25 mM. The thermal profile is: 10 min at 95°C for polymerase activation and 45 cycles consisting in 20 s at 95°C and 1 min at 60°C. Reaction volume contains 20 μl of mix solution and 5 μl of DNA eluate. The real-time PCR for *T. equi* amplifies an 81-bp fragment outside the V4 hyper variable region of 18S rRNA gene. Primers (F: Be18SF; R: Be18SR) at 0.9 μM and TaqMan probe (VIC-TAMRA, Be 18SP) at 0.25 mM were employed as reported by Kim et al. (2008) [17]. The thermal profile used was: 10 min at 95°C and 45 cycles constituted by 20 sec at 95°C and 1 min at 55°C. Reaction volume contains 20 μl of mix solution and 5 μl of eluate. Together with the primers and probe, the mix solution contains 12.5 μl of TaqMan 2X PCR Master Mix (Applied Biosystems, ThermoFisher Scientific, Waltham, MA USA) and a volume of UltraPure™ DEPC-Treated Water (Applied Biosystems, ThermoFisher Scientific, Waltham, MA USA) to reach the final reaction volume of 20 μl.

A CBC was performed by the automated counter Cell-Dyn 3700 (Abbott, Lake Forest, Illinois, USA). Serum samples were analysed by the automated analyser Olympus AU400 (Olympus, Tokyo, Japan). The electrophoresis of serum protein was performed by the G26 system (Interlab, Rome, Italy).

Group definitions

The first step of data analysis was a verification on the suspect cases form (see Fig. 1), excluding those subject not fitting the case definition. Successively, suspect cases were classified as confirmed cases (Group A) or non-confirmed suspects (Group C), based on serological and biomolecular results. Signs that affect laboratory parameters, such as anaemia and jaundice, were verified by CBC and total and direct bilirubin values respectively.

Being signs often not confirmed by laboratory results, the actual presence of haematuria and petechial haemorrhages was brought into question and a modified suspect case definition was set as: temperature > 39°C and presence of at least one laboratory confirmed sign among anaemia and jaundice.

Considering the long persistence of antibodies against EP in infected animals, in particular in the case of *T. equi* infection, the serological positivity although in presence of compatible signs as not considered sufficient to define an acute case of piroplasmosis, thus, a suspect case as confirmed only if real-time PCR was positive.

Group B consisted of confirmed controls (healthy) after excluding controls resulted positive, even if asymptomatic. Non confirmed suspect and suspect cases with positivity in real-time PCR for other diseases, as leptospirosis, equine infectious anaemia and anaplasmosis were included into Group C.

Statistical analysis

Anamnestic data were considered as exposure variables and were reported as absolute frequencies and percentages (%). Age variable (in years) was categorized in three classes following the quantile distribution: from 0 to 6 years, from 7 to 12, and over 13 years. The agonistic life of the horses, in case of sportive ones, is included completely in the first category (0–6); after that the horse could be employed both for reproductive and recreational purposes. Univariate logistic regressions were performed using groups (A vs B and A vs C) as outcome and anamnestic data as independent variables. The significant variables in univariate regressions were combined in multivariable logistic analysis as independent variables. ORs with 95% confidence intervals (CI) were estimated.

Haemato-biochemical parameters were reported as medians and interquartile ranges (IQR = 75°-25° percentile). Differences among groups for each parameter were evaluated by Kruskal-Wallis H-test (H), followed, when significant, by Bonferroni correction for multiple comparisons. A P value < 0.05 was considered statistically significant. SPSS v.27 (IBM SPSS Statistics) were used for all statistical analyses.

Results

The selection pathway is displayed in the flow-chart of Fig. 1. A total of 253 suspect cases were excluded for not correctly fitting the acute case definition. Group A, confirmed cases, was composed by 19 *T. equi* PCR-positive animals, 16 horses and three donkeys. Percentages of confirmed cases showing each sign present on the submission form are displayed in Fig. 2, compared to those reported by Risso et al. (2022) [18], Laus et al. (2015) [19] that involves donkeys, Al Saad et al. (2010) [20], Zobba et al. (2008) [21], and Hailat et al. (1997) [22]. One hundred and two horses and 19 donkeys composed Group B, and 23 horses and four donkeys were included in group C.

Regarding the positivity to other diseases, among samples belonging to group A one horse was serologically positive for *Leptospira spp.*, and another one to IFAT for *Anaplasma phagocytophilum*; group B included five samples serologically positive for *Leptospira spp* and three positive to IFAT for *A. phagocytophilum*, and finally in group C two samples were also serologically positive to *A. phagocytophilum.* A further seven and 21 subjects were serologically positive to *Leptospira spp.* and *A. phagocytophilum* respectively, but they were excluded for one of the reason reported in Fig. 1.

No DNA of both *Leptospira spp*. and *A. phagocytophilum* was detected. Moreover, all samples were negative to equine infectious anaemia.

Results of the univariate analysis on the individual and management characteristics are showed in Table 3. Anamnestic variables that influenced the probability to belong to group A versus first control group were age, breed, recent introduction, housing, vaccination and ectoparasiticide treatment. Age (OR = 7.20 [95% CI 1.46–35.28] for 7–12 years and OR = 7.46 [95% CI 1.49–37.41] for ≥ 13 years) recent introduction (OR = 22.31 [95% CI 2.19-227.63]) and outer housing (OR = 4.21 [95% CI 1.53–11.58]) variables were significant risk factors, instead galopper breed (OR = 0.08 [95% CI 0.01–0.95]), vaccination (OR = 0.04 [95% CI 0.00-0.43]) and ectoparasiticide treatment (OR = 0.15 [95% CI 0.03–0.68]) were significant protective factors. No risk differences were detected between group A and group B respect to other variables.

Table 3
Results of the logistic univariable analysis on individual anamnesis and management parameters collected with the anamnestic form.

Individual anamnesis		Groups			Logistic univariable analysis			
Variable	Outcome	Cases (A)	Control (B)	Not confirmed suspects	A vs B		A vs C	
		[N = 19]	[N = 121]	[N = 27]	OR	95% CI	OR	95% CI
Species	Horse	16 (84%)	102 (84.3%)	23 (85.2%)				
	Donkey	3 (16%)	19 (15.7%)	4 (14.8%)	1.00	0.27–3.79	1.01	0.21–5.49
Gender	Male	2 (11%)	24 (19.8%)	0				
	Gelding	11 (58%)	38 (31.4%)	15 (55.6%)	3.47	0.71–17.05		
	Female	6 (32%)	59 (48.8%)	11 (40.7%)	1.22	0.23–6.47		
	Missing	0	0	1 (3.7%)				
Age (y)	0–6	2 (11%)	56 (46.3%)	2 (7.4%)				
	7–12	9 (47%)	35 (28.9%)	4 (14.8%)	**7.20***	**1.46–35.28**	2.25	0.23–22.14
	>13	8 (42%)	30 (24.8%)	20 (74.1%)	**7.46***	**1.49–37.41**	0.4	0.04–3.34
	Missing	0	0	1 (3.7%)				
Fur colour	Light	11 (58%)	48 (39.7%)	18 (66.7%)				
	Dark	8 (42%)	71 (58.7%)	9 (33.3%)	0.49	0.18–1.31	1.45	0.43–4.88
	Missing	0	2 (1.7%)	0				
Breed	Autochthonous	2 (11%)	2 (1.7%)	3 (11.1%)				
	Saddler	10 (53%)	65 (52%)	18 (66.7%)	0.15	0.02–1.22	0.83	0.12–5.85
	Trotter	1 (5%)	10 (8%)	0	0.10	0.01–1.71		

Individual anamnesis		Groups			Logistic univariable analysis			
	Galloper	2 (11%)	24 (19.8%)	0	**0.08***	**0.01–0.95**		
	Other	1 (5%)	1 (0.8%)	2 (7.4%)	1	0.03–29.81	0.75	0.04–14.97
	Missing	3 (16%)	19 (15.7%)	4 (14.8%)				
Ticks	**No**	12 (63%)	54 (43.2%)	15 (55.6%)				
	Yes	7 (37%)	65 (52%)	10 (37%)	0.48	0.17–1.31	0.87	0.26–2.99
	Missing	0	2 (1.6%)	2 (7.4%)				
Management		**Groups**			**Logistic univariable analysis**			
Variable	**Outcome**	**Cases (A)**	**Control (B)**	**Not confirmed suspects**	**A vs B**		**A vs C**	
		[N = 19]	**[N = 121]**	**[N = 27]**	**OR**	**(95% CI)**	**OR**	**(95% CI)**
Recent Introduction	**No**	16 (84%)	119 (98.4%)	27 (100%)				
	Yes	3 (16%)	1 (0.8%)	0	**22.31****	**2.19-227.63**		
	Missing	0	1 (0.8%)	0				
Housing	**Inner**	7 (37%)	86 (71.1%)	15 (55.6%)				
	Outer	12 (63%)	35 (33.9%)	11 (40.7%)	**4.21****	**1.53–11.58**	2.33	0.69–7.87
	Missing	0	0	1 (3.7%)				
	Single	10 (53%)	78 (64.5%)	9 (33.3%)				
	Group	5 (26%)	31 (25.6%)	4 (14.8%)	1.67	0.55–5.10	1.50	0.31–7.18
	Missing	4 (21%)	12 (9.9%)	14 (51.9%)				
Vaccination	**No**	3 (16%)	1 (0.8%)	6 (22.2%)				

Individual anamnesis		Groups			Logistic univariable analysis			
	Yes	15 (79%)	119 (98.4%)	21 (77.8%)	**0.04****	**0.00-0.43**	1.42	0.31–6.64
	Missing	1 (5%)	1 (0.8%)	0				
Ectopar. treatments	**No**	15 (79%)	64 (52.9%)	19 (70.4%)				
	Yes	2 (11%)	57 (47.1%)	7 (25.9%)	**0.15***	**0.03–0.68**	0.36	0.065-2.00
	Missing	2 (11%)	0	1 (3.7%)				
Deworming	**No**	1 (5%)	1 (0.8%)	1 (3.7%)				
	Yes	16 (84%)	120 (99.2%)	26 (96.3%)	0.13	0.01–2.23	0.62	0.04–10.54
	Missing	2 (11%)	0	0				
Deworming periodicity	**Regular**	13 (68%)	95 (79%)	3 (11%)				
	Sporadic	4 (21%)	25 (20%)	3 (11%)	1.17	0.35–3.89	3.38	0.54–21.11
	Missing	2 (11%)	1 (1%)	21 (78%)				
*p < 0.05, **p < 0.01. Significant OR and 95% CI highlighted in bold								

According to multivariate analysis results, age variable was a significant risk factor (OR = 11.80 for 7–12 years [95% CI 2.18–63.83] and OR = 18.70 for ≥ 13 years [95% CI 3.39–103]), while ectoparasiticide treatment (OR = 0.06 [95% CI 0.01–0.31]) was a significant protective factor.

Regarding to haemato-biochemical parameters, results of statistical analysis are reported in Table 4. Three sets of parameters could be identified. The first set includes the parameters that did not vary significantly among group A (confirmed cases) and group B (control group) such as AST, GGT, WBC, segmented and band neutrophils, lymphocytes (both as absolute and percentage values), MCV, MCHC, total proteins, particulary alfa and beta seroprotein fractions. The second set gathers the parameters that are significantly different between group A and B (control group) even though not between group A and group C (not confirmed suspects). These are total and direct bilirubin, monocytes, basophils, MCH and gamma fraction, that have median values higher in group A compared to groups B. CK, eosinophils (both as absolute and percentage values), RBC, HGB, HCT, RDW, albumin, and A/G ratio have median values lower in group A

compared to groups B. Ultimately, the platelet count is the only parameter that shows a significant difference between groups A and both the other groups (Table 4).

Table 4
Comparisons of haemato-biochemical parameters among groups: confirmed cases (A), controls (B), and not-confirmed suspects (C).

Haematobiochemical parameters	Cases (A)	Control (B)	Not confirmed suspects (C)	H test [a], p value	Multiple comparisons
	[N = 19]	[N = 121]	[N = 27]		
	Median (IQR)	Median (IQR)	Median (IQR)		
AST	248 (110)	303 (105)	290 (176)	H = 5.71, P = 0.06	
Direct bilirubine	0.72 (0.75)	0.47 (0.20)	0.54 (0.56)	H = 14.33, **P = 0.001**	**A vs B****
Total Bilirubine	4.95 (7.34)	1.69 (0.81)	1.74 (4.68)	H = 9.87, **P = 0.007**	**A vs B****
CK	169 (158)	277 (149)	188 (132)	H = 26.37, **P < 0.001**	**A vs B***, B vs C****
GGT	14 (16)	13 (10)	16 (43)	H = 1.09, P = 0.578	
WBC	8.03 (4.52)	7.36 (2.53)	7.73 (2.62)	H = 2.93, P = 0.231	
Neutrophils	5.13 (2.53)	4.23 (1.64)	5.59 (2.80)	H = 5.99, P = 0.06	
Segmented neutrophils (%)	69 (19)	60 (11)	70 (13)	H = 12.44, **P = 0.002**	**B vs C****
Band cells (%)	0 (0)	0 (0)	0 (0)	H = 4.02, P = 0.134	
Lymphocytes	1.84 (1.62)	2.18 (1.26)	1.52 (1.04)	H = 13.99, **P = 0.001**	**B vs C****
Lymphocytes (%)	25 (14)	35 (11)	20.5 (15)	H = 19.81, **P < 0.001**	**B vs C*****
Monocytes	0.90 (1.36)	0.34 (0.18)	0.42 (0.21)	H = 26.04, **P < 0.001**	**A vs B***, B vs C****
Monocytes (%)	3 (5)	0 (0)	0 (4)	H = 21.48,**P < 0.001**	**A vs B***
Eosinophils	0.10 (0.22)	0.22 (0.23)	0.24 (0.35)	H = 8.50, **P = 0.014**	**A vs B***
Eosinophils (%)	2 (3)	3 (3)	2.5 (7)	H = 6.65, **P = 0.036**	**A vs B***

Haematobiochemical parameters	Cases (A)	Control (B)	Not confirmed suspects (C)	H test [a], p value	Multiple comparisons
	[N = 19]	[N = 121]	[N = 27]		
	Median (IQR)	Median (IQR)	Median (IQR)		
Basophils	0.05 (0.06)	0.03 (0.02)	0.03 (0.03)	H = 10.22, **P = 0.006**	**A vs B****
Basophil (%)	0 (0)	0 (0)	0 (0)	H = 3.17, P = 0.204	
RBC	4.67 (1.38)	7.32 (1.53)	5.415 (1.03)	H = 52.8, **P < 0.001**	**A vs B***, B vs C****
HGB	8.56 (3.66)	12.50 (1.70)	9.75 (2.49)	H = 55.58, **P < 0.001**	**A vs B***, B vs C****
HCT	24.7 (11.7)	36.5 (4.9)	29.3 (6.9)	H = 52.65, **P < 0.001**	**A vs B***, B vs C****
MCV	50.1 (5.8)	49.5 (5.8)	54.2 (6.2)	H = 17.31, **P < 0.001**	**B vs C****
MCH	17.5 (1.6)	16.8 (1.6)	18.6 (2.1)	H = 20.98, **P < 0.001**	**A vs B*, B vs C****
MCHC	34.7 (1.3)	34.1 (1)	33.65 (0.90)	H = 14.17, **P < 0.001**	**B vs C*, A vs C****
RDW	22.5 (2.2)	25 (2)	23.2 (1.6)	H = 31.81, **P < 0.001**	**A vs B***, B vs C****
PLT	42.4 (66.1)	115 (48.4)	126.5 (61.7)	H = 16.71, **P < 0.001**	**A vs B***, A vs C****
Total protein	6.4 (1.1)	6.3 (0.6)	6.35 (0.6)	H = 0.39, P = 0.819	
Albumin	2.66 (0.44)	3.02 (0.24)	2.74 (0.44)	H = 34.96, **P < 0.001**	**A vs B***, B vs C****
Alfa 1	0.09 (0.03)	0.1 (0.01)	0.1 (0.03)	H = 0.91, P = 0.634	
Alfa 2	0.73 (0.24)	0.81 (0.13)	0.85 (0.33)	H = 4.24, P = 0.120	
Beta	0.73 (0.23)	0.68 (0.20)	0.69 (0.25)	H = 1.84, P = 0.398	
Gamma	1.98 (0.61)	1.58 (0.51)	1.84 (0.64)	H = 17.78, **P < 0.001**	**A vs B**, B vs C***

Haematobiochemical parameters	Cases (A)	Control (B)	Not confirmed suspects (C)	H test [a], p value	Multiple comparisons
	[N = 19]	[N = 121]	[N = 27]		
	Median (IQR)	Median (IQR)	Median (IQR)		
A/G ratio	0.73 (0.29)	0.94 (0.19)	0.755 (0.23)	H = 33.05, **P < 0.001**	**A vs B***, B vs C***

[a]H test: Kruskal-Wallis test, *df* = 2; *p < 0.05, **p < 0.01, ***p < 0.001. IQR: interquartile range. Significant P highlighted in bold.

AST: aspartate aminotransferase, CK: creatinphosphokinase, WBC: total white blood cells, RBC: total red blood cells count, HGB: haemoglobin level, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, RDW: red cell distribution width, PLT: platelets, A/G ratio: Albumin/Globulin ratio

Discussion

EP is widely present in many countries of the world, so much so Schnittger et al. (2012) [23] estimated that around 90% of the horses live in endemic areas. Thus EP should always to be considered in the differential diagnosis, together with other diseases such as leptospirosis, equine infectious anaemia and anaplasmosis, in case of any anaemic/haemolytic clinical forms, as presence of anaemia, jaundice or petechiae. This study was set with the aim of investigating if individual and management characteristics could influence the probability of being infected. Furthermore it was assessed the possible association of some haemato-biochemical alterations with confirmed cases *of T. equi* infection, comparing them to healthy subjects and to those ones with compatible signs but not confirmed as *T. equi* infected. The original purpose was to investigate both EP agents but, since only one case of *B. caballi* was detected among the subjects eligible, the study was focused on *T. equi*.

Symptoms are not often reported in details in the studies on EP. Comparing the present study with the few others for which clinical data are reported or easily computable (Fig. 1) a variability in the presence of symptoms emerges, with the predominance (presence in ≥ 50% of confirmed cases) of anaemia, jaundice, lack of appetite, lethargy, petechial haemorrhages, weakness and weight loss.

Gender and species have no influence on the presence of *T. equi*, while age is a risk factor for *T. equi* and other diseases, with a cumulative age-dependent increasing presence of the pathogen. Positive cases increase in the groups 7–12 and > 13 years of age compared to the 0–6 years group. This result was expected and in agreement with previous studies [24], even though it disagrees both with the results of a study by the authors of the same regions [4], reporting that OR decreased with age, and with Steinman et al. (2012) [25] that, in Israel, observed no significant differences within the age classes considered. This evidence could be due to the cumulative risk of being in contact with an infected ticks through the life, to

the inefficacy of the treatment, that can be variable depending on the strain involved [26] and to the fact that *T. equi* is not self-cleared as *B. caballi* but leads to a chronic infection status.

Breed analysis highlighted a lower risk (OR = 0.08, [95% CI 0.01–0.95]) to be infected in the galopper breed compared to others. It could be a proxy related to different management practices, according to breed and attitude, i.e. more appropriate rearing conditions of pure breed horses and of those ones with economical value, that resulted less exposed to ticks [25, 27]. Because of the sampling was carried out almost only amongst animals belonging to saddler breed and with equestrian attitude, despite authors recommendations to practitioners, no conclusions can be certainly inferred.

The assessment of the fur brightness was included in the study as well as previously considered by Katiyatiya et al. (2015) [28] in the bovine, assuming that dark colour furs could result in a higher tick load and, consequentely, in an increased risk of infection. However, the present survey is still at a preliminary stage and further studies will be needed to clarify a possible association between T. equi infection and fur colours.

T. equi confirmed cases were approximately four times more likely to be found amongst animals having an external access compared to controls, reinforcing the evidence that access to the outside, including not only a paddock near the stable but also pastures and free-ranging animals, in which the infection risk could be higher for a greater exposure to ticks or for a lack of grooming practices that improve tick removal, is a significant variable, in agreement with previous studies [25, 29, 30].

Vaccinations against other equine diseases and ectoparasiticide treatment seems to have a protective effect on *T. equi* infection. While for the latter variable the association with a lower presence of infection is expectable, the correlation with vaccination is not clear; it is opinion of the authors that this evidence could be explained with prophylactic treatments being a proxy for good management practices that prevent the infection.

Several studies on haemato-biochemical parameters associated with EP are available for both horses and donkeys. In the present study, the authors performed a comparison of haemato-biochemical parameters among the three groups. AST value resulted lower in the case group, even if not statistically significant (p = 0.06). This finding differs from previous studies [20, 22, 31, 32] reporting an increase, even if not constant, of this enzyme in acute cases as well as for muscle enzyme CK. Both total and direct bilirubin values resulted higher in cases compared to controls, as well as reported by several other studies [19–22, 31], whereas, in the unconfirmed suspects, bilirubin values resulted between those of the other two groups. However only direct birilubin values were over the reference value. A decrease in RBC count, HGB and HCT confirmed *T. equi* associated findings, caused by the haemolysis of the red blood cells [19–24, 31, 25–28]. The decrease in platelet count is widely reported [20, 21, 31–35], and it is the only parameter that, according to the present data, differs between both cases and controls rather than between cases and non confirmed suspect (Fig. 3). This statistical evidence though, seems to lack any biological explanation justifying it, if compared to other similar disease as *B. caballi*. In other species, such as dogs with babesiosis, the platelet count decrease is supposed to be caused by sequestration in the spleen, systemic

disseminated intravascular coagulation, or immune-mediated depletion [36]. An increase of MCH is also reported in donkeys [19, 33] and horses [35] while variation in MCV, as an increased value, is reported only by Davitkov et al. (2017) [33] and Mahmoud et al. (2016) [35] and as a decreased value only in two out of 16 cases by Pasolini et al. (2018) [33]. In the present study, MCV resulted different only between controls and unconfirmed suspects. MCHC is reported to vary in affected donkeys [19, 33] whereas in the present study resulted not significantly different, as well as for others [20–22, 31, 34, 35]. An increase of WBC count was not observed in the present study despite being reported in several others [18, 20–22, 31, 33, 34] as well as a decrease in total protein [20, 22]. This is the first report describing a decrease in eosinophils, basophils and RDW associated to *T. equi* infection, in contrast with Mahmoud et al. (2016) [35] but in agreement with previous studies in cattles with theileriosis [37]. Hypoalbuminemia and hyperglobulinemia were observed with a consequent decrease of the ratio of the two parameters. Usually albumin decreases as a result of liver dysfunction due to the disease. A decrease in albumin was reported also in dogs with babesiosis [38], but this is the first finding in infected equids, following Zobba et al. (2008) [21] that described, without inferential analysis, an albumin decrease in 15% of the positive subjects whereas globulin was reported as normal in all of them; and Pasolini et al (2018) [32] that reported a decreased value in two of 16 positive horses. Finally it has to be highlighted that some of the haemato-biochemical parameters, in particular the total bilirubin, CK, percentage of monocytes, eosynophils, basophils, MCH, RDW, albumin, and gamma fraction, despite being associated to *T. equi*, are still within the reference values. The same observation has been reported in most of the studies previously mentioned in the discussion, meaning that, in evaluating the laboratory results, these parameters are not useful for practioners, that have to refer only to meaningful parameters such as direct bilirubin, RBC, HGB, HCT and A/G.

Limitations of this study has been already partially discussed in ‘material and methods’ section, as they influence the case definition and data analysis. Interviewer bias brought the major constraint to the study, in fact, in many cases, symptoms reported by the practitioners were not confirmed by the laboratory results, suggesting that the data provided were manipulated to result eligible for the study. The reason could be the following: the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana “M. Aleandri” offered free analysis for the subject enrolled in the study to incentive the collaboration of the equine practitioners, and some of them could have taken advantage of this offer, submitting forms reporting non-observed signs. The double check between signs and laboratory findings aimed to overcome this limitation, excluding subjects with insubstantial data.

The aim of the study, as already mentioned, was to provide valid tools for the clinical activities to the practitioners even if, after discussing the statistical and the construct validity, external validity should be discussed, i.e. whether the study results can be generalized to different settings and times [39], as other species, breeds and attitudes, and different level of prevalence. Moreover, a similar investigation should be performed also for *B. caballi* and other diseases in the differential diagnosis of anemic/haemolotic clinical form to provide more data in order to assess a more precise diagnosis.

Conclusions

The main conclusion highlighted by the study is that any investigated haemato-biochemical parameters, except platelet count, seem to be irrevocably useful to discriminate *T. equi* from other diseases. The present study confirms that the diagnosis of *T. equi* could be an issue, for its similarities with other diseases, but it still represents a starting point for further investigations, highlighting unsolved critical points and providing the practitioners with further data for the interpretation of laboratory results. Moreover, this study focuses the attention on prevention practices, as external antiparasitic treatment, the control of access to pastures and good management practices in general, that together with regular laboratory controls, even in asymptomatic animals, can reduce the spread of the disease.

Abbreviations

A/G: Albumin globulin ratio; AST: aspartate aminotransferase; CBC: complete blood cell count CK: creatine phosphokinase; EDTA: ethylenediaminetetraacetic acid; ELISA: enzyme-linked immunosorbent assay; EP: equine piroplasmosis; GGT: gamma-glutamyltransferase; HCT: haematocrit; HGB: haemoglobin; IFAT: indirect fluorescent antibody test; MCH: mean corpuscolar haemoglobin; MCHC: mean corpuscolar haemoglobin concentration; MCV: mean corpuscolar volume; OIE: World Animal Health Organization; OR: Odds Ratio; PCR: polymerase chain reaction; PLT: platelets count; RBC: red blood cells; RDW: red blood cells Distribution Width; WBC: total white blood cells.

Declarations

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Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RN, FR and MTS concept and designed the study; RN, AC, FR, OL, GB, MRV and AA performed laboratory analysis; RN and FI performed statistical analyses; RN and FI drafted the work; RN, FI, FR, PS, OL, GB and MTS revised the manuscript.

Ethics approval and consent to participate

This article does not contain any studies on animals performed by any of the authors. Veterinary practitioners collected blood samples during their clinical activities for diagnostic purposes.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Endnotes

a:http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.05.08_EQUINE_PIROPLASMOSIS.pdf

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Figures

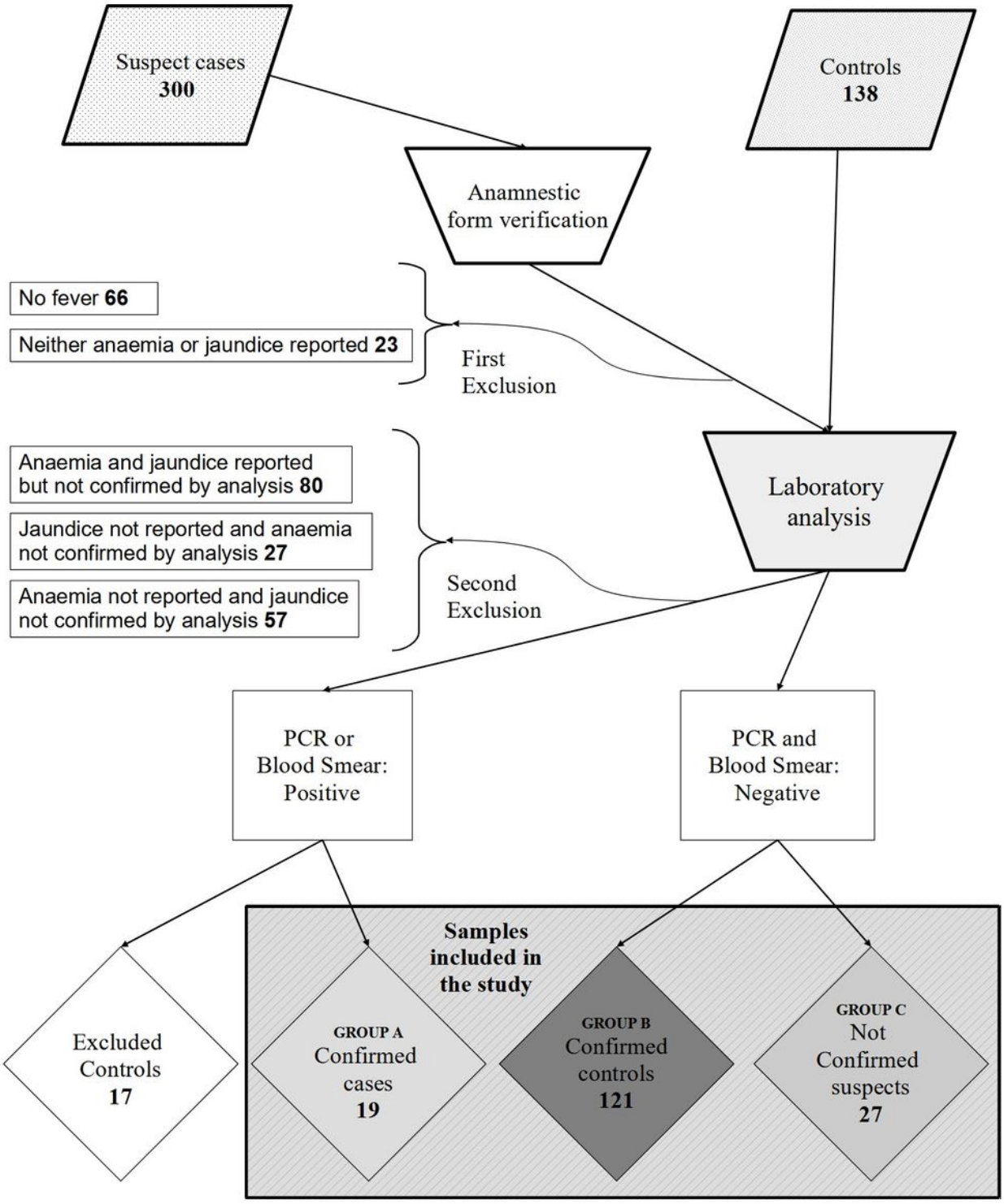

Figure 1

Flow chart representing the selction path followed by suspect cases and controls samples and final number of each category included in the statistical analysis.

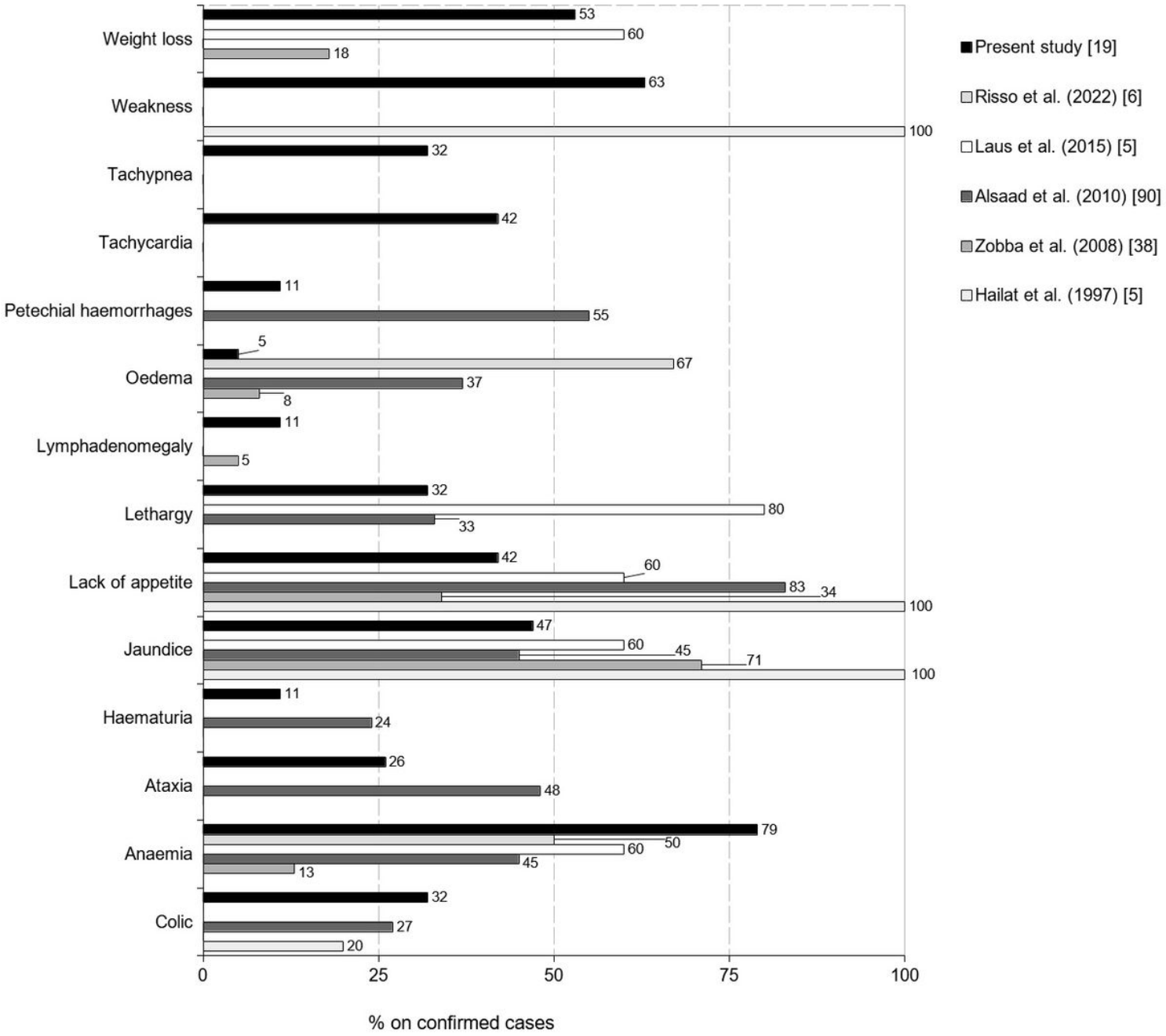

Figure 2

Percentages of confirmed cases presenting each of the sign included in the anamnestic data collection form (black bars) compared with those reported by Risso et al. (2022) [18], Laus et al. (2015) [19], Al Saad et al. (2010) [20], Zobba et al. (2008) [21], and Hailat et al. (1997) [22]. Numbers in square brackets represent the total number of confirmed cases of each study.

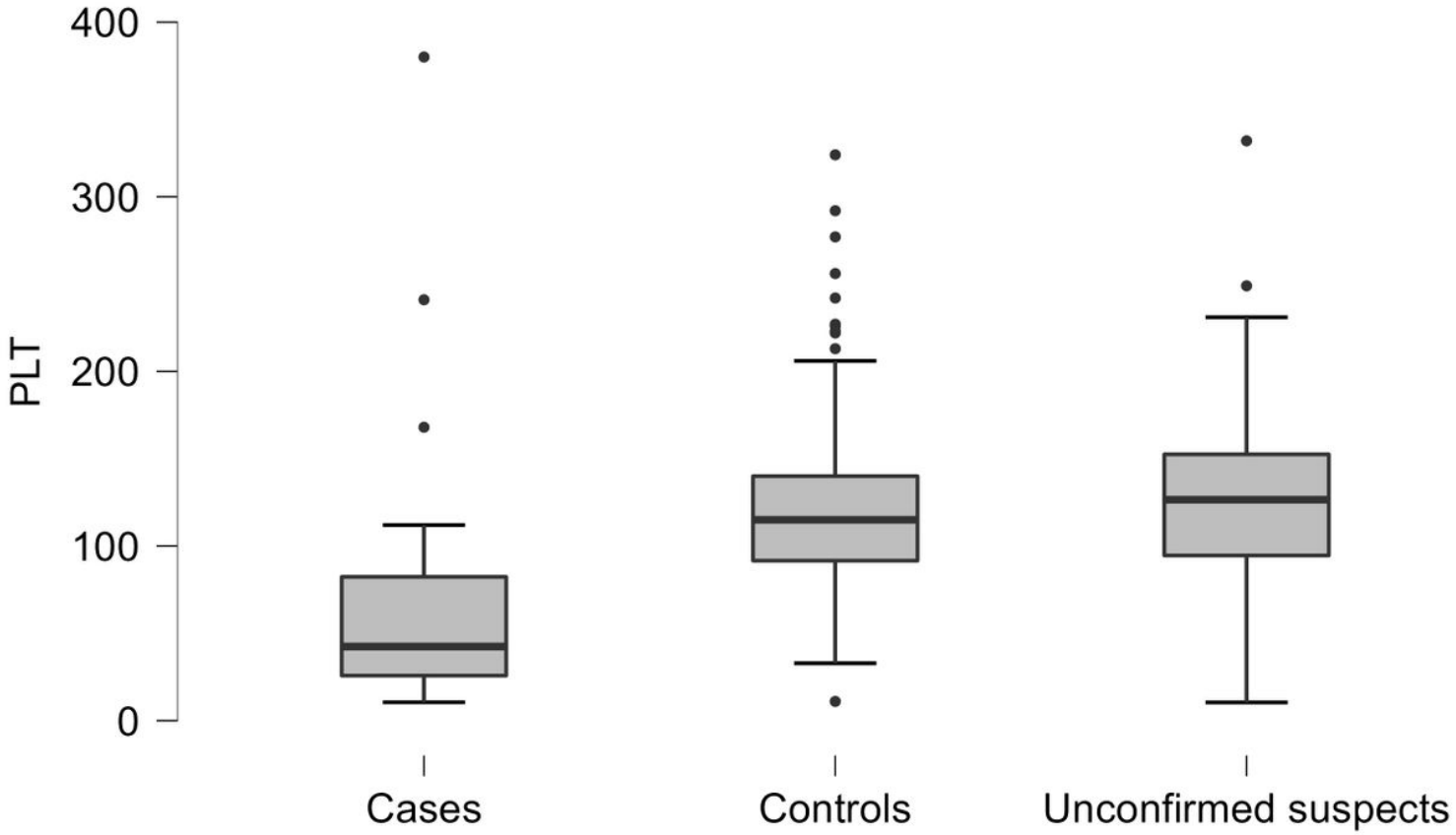

Figure 3

Boxplot of PLT values in the three groups evaluated in the study