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Original article

Comparison of direct and indirect methods to maximise the detection of *Babesia caballi* and *Theileria equi* infections in Central Southern Italy

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ABSTRACT

Equine piroplasmosis is a disease of equids, caused by tick-borne apicomplexan protozoan pathogens *Babesia caballi* and *Theileria equi*, which, according to the World Organisation for Animal Health (OIE), can be diagnosed by enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody test (IFAT) and polymerase chain reaction (PCR). The present study was conducted to evaluate and compare the assays available for the diagnosis of equine piroplasmosis. Data employed were obtained from 1300 blood samples collected between 2012–2014 from asymptomatic and symptomatic equines (horses and donkeys) of central-southern regions of Italy and analyzed by ELISA, IFAT, PCR (one commercial and one from literature) and blood smear microscopic examination. Statistical differences of the proportions of positivity for each parasite and group (asymptomatic and symptomatic) among the methods were verified by the z test to identify the most sensitive. The concordance between each pair of methods – for each parasite and within the groups – and trends in detection of suspect samples of four hypothetical diagnostic algorithms using serological and biomolecular assays were evaluated to identify the most suitable laboratory diagnostic workflow.

The results of this study highlighted a lower capacity to detect suspect samples of commercial ELISA for *B. caballi* in all groups when compared to biomolecular methods and IFAT; and of the commercial PCRs in asymptomatic animals, identifying a PCR from literature and IFAT as the best choice for a combined diagnosis. For *T. equi,* IFAT detected more suspect samples than ELISA, even if the latter showed good performance and some samples were positive only by the ELISA and PCR, indicating that their simultaneous employment is still advantageous. Host-parasite interaction, amino-acid/genetic diversity and differences in detection limits among the assays could be among the reasons in explaining the present results.

In view of further studies, ELISA should be used in combination with PCR, that should regularly be included in the laboratory diagnosis to maximise the detection of early infections and support the evaluation of pharmacological treatment.

1. Introduction

Babesia caballi and *Theileria equi* are two intra-erythrocytic apicomplexan protozoa, aetiological agents of a "tick borne" disease known as equine piroplasmosis (EP). EP affects horses, mules, donkeys and zebras, and is endemic in many countries in tropical and temperate areas. Both parasites are transmitted by ticks belonging to six genera: *Dermacentor, Haemaphysalis, Hyalomma, Ixodes, Rhipicephalus* and *Amblyomma*; with *Hyalomma, Dermacentor* and *Rhipicephalus* most frequently involved in the transmission (Scoles and Ueti, 2015). EP is also a major constraint to the *international* movement of horses and a possible cause of heavy economical losses to the horse industry (Friedhoff et al., 1990; Sumbria et al., 2017; Onyiche et al., 2019).

The disease can occur in three clinical forms: acute, subacute and

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chronic. Typical clinical signs of EP include: fever, depression, anaemia, jaundice, oedema, anorexia and occasionally mucosal petechiae and ecchymoses (Sumbria et al., 2014). As symptoms are similar for both protozoa, differential diagnosis exclusively based on clinical signs is not reliable (de Waal, 1992; Sumbria and Singla 2015), even if infection with *B. caballi* is usually clinically milder.

A relevant difference that exists between the two equine piroplasms, is to be underlined: *B. caballi* infection can be self-cleared by the host (Wise et al., 2014), with the disappearance of antibodies (Schwint et al., 2009) while infection with *T. equi*, if untreated, leads to a chronic asymptomatic carrier status with low levels of parasitaemia and life-long persistence of antibodies (de Waal, 1992; Ueti et al., 2012).

In the view of the fact that around 90% of the world horse population lives in endemic areas (Schnittger et al., 2012) and persistently infected asymptomatic equids represent the main risk factor for the spread of EP (Lobanov et al., 2018), laboratory diagnosis should be a mandatory requirement to avoid introduction of infected animals in EP-free countries such as Canada, New Zealand, Australia, and Singapore (Brüning, 1996; Rothschild, 2013). Within the European Union, for introduction and movement among the different countries, laboratory testing for EP is not mandatory as only a clinical evaluation of absence of symptoms of any transmissible disease is required as proof of freedom from infection (Council Directive 2009/156/EC).

Several laboratory tests can be performed for EP diagnosis using examination of stained blood smears, serological tests, such as complement fixation test (CFT), indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunoblot (IB). In addition, biomolecular methods with various types of PCR assays are available (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2018a).

The accuracy and sensitivity of the thin or thick blood smear tests are low especially in asymptomatic animals and rely on the experience of the reader and the number of infected red blood cells (RBC). In fact, even during the acute phase of the disease, it is extremely difficult to demonstrate the presence of EP parasites as only one out 10^3 RBCs could be infected (Rothschild and Knowles, 2007).

Serological assays were rapidly developed throughout the years and the first test to be implemented was the CFT (Hirato et al, 1945), followed by the introduction of IFAT (Ristic and Sibinovic, 1964) that is more sensitive than CFT and was initially reported as capable of distinguishing between the two piroplasms (Madden and Holbrook, 1968). Actually, as indicated by the manufacturers of commercial assays, cross reactivity is possible and to distinguish between the two infections, further testing with the scalar dilution of the sera is required, with consequent time and reagents consume. Nevertheless, since 2005, together with the ELISA, IFAT is one of the tests for EP recommended by the OIE (2018a). ELISA is characterized by a higher throughput of samples and a reduced turn-around time compared to the IFAT and could be easily standardized with possible automation. A competitive-ELISA (c-ELISA) for each parasite is commercially available in Italy: *B. caballi* c-ELISA uses a recombinant rhoptry-associated protein 1 (rap-1) as antigen while *T. equi* c-ELISA employs a recombinant Equi Merozoite Antigen 1 (ema-1). Sensitivity and specificity of these c-ELISAs reported by the manufacturers are, respectively, 100% and 100% for *B. caballi* and 95% and 99.5% for *T. equi* while Sumbria et al. (2015) reported for the same assays 91% and 70% for *B. caballi* and 96% and 95% for *T. equi*, respectively.

Recently, an IB assay detecting antibodies against EMA-1 and EMA-2 was developed to verify antibody clearance after treatment for *T. equi* infection (Wise et al., 2018).

Several PCR methods are available for EP diagnosis, different for type (End-point, Nested, real-time PCR) and target (18S rRNA or Bc48 for *B. caballi*; EMA-1 or EMA-2 gene for *T. equi*) (Battsetseg et al., 2001; Nicolaiewsky et al., 2001; Kim et al., 2008, Bhoora et al., 2010a). Molecular assays capable of simultaneously detecting both parasites and identifying the prevalent agent of infection were recently developed (Bhoora et al., 2018; Montes Cortés et al., 2019; Lobanov et al., 2018).

PCRs are very sensitive and specific but do not vouch for a parasitic negative status, as sporadic negative PCR results were reported from experimentally infected horses (Grause et al., 2013). Moreover, the definition of a cut-off threshold and interpretation of borderline Cts can be challenging (Alanazi et al., 2014).

A correct definition of the infection status is crucial to prevent the spread of the disease and to avoid unnecessary treatments. As the Gold Standard test (GS) for EP diagnosis is not available, this study was conducted to evaluate and compare, solely in terms of concordance, the performances of serological and molecular assays for the detection of EP infection, both in asymptomatic and asymptomatic equids with the aim to also evaluate if the carrier status has a different pattern of laboratory results as well as to identify the most suitable protocol to satisfy the diagnostic needs of practitioners in detecting, preventing, controlling and treating EP.

2. Materials and methods

2.1. Samples

Laboratory data used for the present study, conducted at the National Reference Laboratory for Equine Diseases (NRL-ED), were obtained from the laboratory results on 1300 blood samples collected between 2012 and 2014 from horses (993) and donkeys (307), residing in Latium, Tuscany, Campania and Molise, Regions of Central-Southern Italy. Laboratory data were collected both retrospectively than from sample collected for the specific purpose of the comparison.

According to the medical history reported by the veterinary practitioners the animals were classified as asymptomatic ($n = 990$ (76.2%); 769 horses and 221 donkeys) or symptomatic ($n = 310$ (23.8%); 224 horses and 86 donkeys), the latter will be referred to as “suspect” in the rest of the manuscript. The definition of suspect was set as the following: $t > 38$ °C (100.4°F) and at least one of the following signs: jaundice, anaemia or petechiae, as reported by Camino et al. (2019). Asymptomatic animals were sampled for research purposes both on the same farms of the symptomatic ones as well as on farms where no EP signs had been reported since at least one year.

2.2. Diagnostic assays

Differential laboratory diagnosis in case of jaundice and anaemia included leptospirosis, anaplasmosis and equine infectious anaemia. These diseases were excluded by micro agglutination test and PCR for leptospirosis (Faine et al., 1999; Ahmed et al., 2009); IFAT (*A. phagocytophilum* IFA IgG Antibody Kit; Fuller Laboratories®, CA, USA) and PCR (Massung et al., 1998) for anaplasmosis and ELISA for equine infectious anaemia (Nardini et al., 2016).

2.2.1. Blood smears

Thin blood smears from EDTA tubes were air-dried, stained with a rapid Romanowsky type stain (Hemacolor® staining kit, Merck, Germany) following the manufacturer’s instructions and examined for intracellular forms of piroplasms under the 100X oil-immersion objective, using a bright-field microscope (Nikon Eclipse E800, Nikon, Japan). A minimum of 100 microscope fields were observed and the outcome (presence/absence of equine piroplasms) was registered.

2.2.2. Serological assays

Two c-ELISA assays, *B. caballi* Antibody test kit and *B. equi* Antibody test kit (VMRD®, Inc, WA, USA), mentioned in the introduction, and two IFAT kits, *B. caballi* IFA IgG Antibody Kit and *B. equi* IFA IgG Antibody Kit (Fuller Laboratories®, CA, USA) were employed according to the manufacturer`s instructions. Sera were analyzed undiluted with the former assays while diluted 1:80 with the latter. The stained IFAT slides were examined by a fluorescent microscope at a magnification of 400X

and each sample well was evaluated for the visual intensity and fluorescence pattern compared to that of the internal positive and negative controls. Outcome of the serological assays was registered as positive or negative at the dilution examined.

2.2.3. Molecular assays

DNA extraction. Genomic DNA extraction from EDTA whole blood samples was performed using the automated robotic workstation QIAcube HT and the cador Pathogen 96 QIAcube HT Kit (Qiagen®, GmbH, Germany) according to manufacturer`s instructions. DNA was eluated in 150 µl of AVE buffer composed of 0.04% NaN3 in RNAase-free water and stored at –80 °C.

Real-time PCRs for B. caballi and T. equi. A preliminary study was conducted to identify the more sensitive PCR methods among those available in literature and commercially available (Nardini et al., 2021). Among these, the most suitable identified for this study are described as follows.

An in house real-time PCR for *B. caballi* was used amplifying a 95-base-pair (bp) fragment in the V4 hyper variable region of 18S rRNA gene of *B. caballi*. Primers (F: Bc-18SF402; R: Bc-18SR496), used at a concentration of 0.9 µM and TaqMan 5120 MGB TM probe (FAM MGB, Bc-18SP) employed at 0.25 mM, were those reported by Bhoora et al. (2010a). The thermal profile for this PCR is as follows: 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. 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.

For *T. equi*, the in-house Real-time PCR, amplifying a 81-bp fragment outside the V4 hyper variable region of 18S rRNA gene was employed. Primers (F:Be18SF; R:Be18SR) at 0.9 µM and TaqMan probe (VIC-TAMRA, Be 18SP) at 0.25 mM were the concentrations reported by Kim et al. (2008). The thermal profile used as follows: 10 min at 95 °C and 45 cycles constituted by 20 s at 95 °C and 1 min at 55 °C. Reaction volume contains 20 µl of mix solution (identical to that for real-time PCR for *B. caballi)* and 5 µl of eluate.

Babesia caballi 18S ribosomal RNA gene Standard Kit and *T. equi* Equi merozoite antigen 1 (ema-1) gene Standard Kit (Genesig®, Primerdesign Ltd, UK) were also employed. Thermal profile for both is the following: 2 min at 95 °C and 50 cycles constituted by 10 s at 95 °C and 1 min at 60 °C. Reaction volume consists of 20 µl of mix solution and 5 µl of eluate.

The internal positive and negative controls for the PCRs from literature consisted in rtPCR products of *B. caballi* and *T. equi,* respectively obtained from EDTA blood samples of a seropositive symptomatic subject and a seronegative asymptomatic subject, cloned in the plasmid vector PCRII®-TOPO® (Invitrogen, CA, USA), and certified by the World Organisation for Animal Health (OIE) Reference Laboratory for Babesiosis and National Reference Laboratory for *Anaplasma, Babesia, Rickettsia* and *Theileria* (CRABaRT) of the Istituto Zooprofilattico Sperimentale della Sicilia (Palermo, Italy). For the commercial assay, internal controls employed were those provided by the manufacturer.

The real-time PCRs were carried out using ABIPRISM 7900 HT Sequence Detection System (A. Biosystems, CA, USA) and the outcome was positive if a sample presented an expected fluorescent curve shape within the Ct threshold value, that was set at 40 Ct.

Limit of detection (LOD) of the real-time PCRs from literature were estimated amplifying the V4 hyper variable regions of the 18S rRNA gene from the certified positive controls using primers specific for *Theileria* and *Babesia* species (Nagore et al., 2004).

The bands on the agarose gel of the positive controls were retrieved and DNA extracted using QIAquick® PCR Purification Kit (QIAGEN, Germany). Extracted DNA concentrations were measured (as ng/µl) using a BioPhotometer® plus (Eppendorf, Germany) and target concentrations were calculated dividing the DNA concentration by the molecular weight of each target. Ten-fold dilutions were prepared and tested in triplicate, in three independent runs. LOD was assigned as the highest dilution resulting positive in all the replicates of the three runs. The LODs declared on the data sheets of commercial PCRs were accepted without verification.

Specificity was verified testing five samples positive for each of the following parasites, *Trypanosoma brucei evansi, Trypanosoma brucei equiperdum, Trypanosoma vivax, Babesia bovis, Babesia bigemina* plus *B. caballi* or *T. equi* certified positive samples depending on the assay; together with 30 samples belonging to horses showing no symptoms and resulting serologically negative for *T. equi* and *B. caballi,* as well as PCR negative.

2.3. Sequencing

The specificity of the literature referenced rtPCR results was verified by sequencing the amplicons of 44 PCR-positive samples amplifying the hypervariable V4 region of the 18 rRNA gene of both protozoa as described by Nagore et al. (2004). The products were sequenced using an automated sequencer (3500 Genetic Analyzer, Applied Biosystems, CA, USA) and the nucleotide sequences obtained were analyzed using the Genetic Analyzer Sequencing v5.4 (Applied Biosystems, CA, USA). Sequence identity was verified using the Basic Local Alignment Search Tool (BLAST) and comparing the sequences obtained for *B. caballi* and *T. equi* to those available in NCBI GenBank (http://www.ncbi.nlm.nih.gov). Sequenced samples presenting an identity and query coverage ≥98% were considered as confirmed.

2.4. Data analysis

Laboratory results were aggregated and, for each parasite and method, number and percentage of positives were calculated for total number of samples, and for asymptomatic and suspect groups.

Statistical differences between pair of assays of the proportion of positive samples were evaluated using the *Z* test employing only samples analyzed with both methods of each pair. A p-value ≤ 0.05 (two-tailed) was considered statistically significant. XLStat 2011 software for Windows (Addinsoft SARL, France) was used for all statistical analyzes.

Concordance (C) between results of each pair of methods, for each parasite and within the three groups described in the previous paragraph, was calculated with formula (1):

$$C = \frac{(A_{Positive} \cap B_{Positive}) + (A_{Negative} \cap B_{Negative})}{Total_{Analysed}} \quad (1)$$

while concordance on positive samples (Cp), for each parasite and within the three groups was calculated with formula (2):

$$C_p = \frac{A_{Positive} \cap B_{Positive}}{A_{Positive} + B_{Positive} - A_{Positive} \cap B_{Positive}} \quad (2)$$

where $A_{Positive}$ is the number of samples positive in assay A, $B_{Positive}$ is the number of samples positive in assay B, $A_{Positive} \cap B_{positive}$ is the number of samples positive in both assay A and B and $Total_{Analyzed}$ is the total number of samples having a result by both assays.

A more stringent value of acceptable concordance than that of 0.81, proposed as threshold by Landis and Kock (1977), was set at: higher than 0.95.

Four hypothetical diagnostic algorithms – each employing four assays run consecutively and in which only the samples resulted negative to the previous test are analyzed with the following one – were analyzed to assess the increase in the laboratory detection of samples suspected to be positive at the addition of an extra method. The four algorithms are

classified by a capital letter, and the methods are reported in order of execution: A (ELISA, IFAT, Commercial PCR, Literature PCR); B (ELISA, IFAT, Literature PCR, Commercial PCR); C (IFAT, ELISA, Commercial PCR, Literature PCR); D (IFAT, ELISA, Literature PCR, Commercial PCR).

3. Results

3.1. Analysis summary

All the samples ($N = 1,300$) were analyzed by the two ELISAs: 71 were positive for *B. caballi* and 493 for *T. equi*. IFAT for *B. caballi* was performed on 381 samples with 50 positives and on 371 for *T. equi* with 157 positives.

Commercial PCR was carried out on 885 samples for both parasites, 22 resulted positive for *B. caballi* and 265 resulted positive for *T. equi*; while PCR from literature was executed on 541 samples for both parasites, detecting 40 positives for *B. caballi* and 316 positives for *T. equi*.

Blood smears of 417 samples were analyzed and presence of microorganism compatible with piroplasms was registered for 29 samples, of which one collected from an asymptomatic donkey.

LOD for the literature PCRs was equal to 39.67 molecules/μl for *T. equi* and equal to 4.35 molecules/μl for *B. caballi*.

3.2. Proportion of positives detected

Proportions of positives detected by each assay, respectively for the total, the horse and the donkey sample population (named in each table as overall) and, within them, for the asymptomatic and suspect groups are reported in Tables 1–3.

A comparison with the blood smear was not possible as it was difficult to make an accurate identification of the parasite species by microscopic examination. This technique does not always allow the identification of the two organisms, especially in case of mixed infections, due to *B. caballi* and *T. equi* super-imposable morphological and biometrical characteristics, despite that they are defined as large and small form, respectively (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2018a).

3.2.1. Whole sample population (horses and donkeys)

Statistical comparison revealed a significant difference ($p < 0.05$, see Table 1) between IFAT and ELISA for *B. caballi* in all groups, with the former detecting more positives; while no difference was detected for *T. equi*, for which the c-ELISA has comparable performance to that of the IFAT. For both parasites, a statistical significant difference was detected between the PCRs in the overall and in the asymptomatic group, with the literature PCR detecting more positives, while no difference was observed in the suspect group.

3.2.2. Horses population

Relative to the horse sample population, for *B. caballi,* a statistical difference was detected between ELISA and IFAT in the overall group and in the symptomatic group; while a statistical difference between PCRs emerged in the overall group and in the asymptomatic group. For *T. equi* only a statistical difference between PCRs in the overall group and in the asymptomatic group was detected.

3.2.3. Donkeys population

In the donkey sample population for *B. caballi,* a statistical difference was detected between ELISA and IFAT in the overall group and in the symptomatic group, together with a statistical difference between the PCR methods for *T. equi*, both in the overall and the asymptomatic group with the literature PCR detecting more positives.

3.3. Concordance

Values of concordance between each pair of methods in the overall, asymptomatic and suspect groups calculated as total and among positives are presented in Table 4.

Wide unexpected discrepancies were detected for concordance on positives between ELISA and IFAT for *B. caballi* and between the two PCRs in all groups of animals. A low value of concordance for positives between ELISA and IFAT was highlighted (0.34) also for *T. equi,* even if higher than that for *B. caballi*.

3.4. Sequencing

The sequences obtained from the PCR-positive samples (44) were identified as specific for either one of the equine piroplasms all having a sequence identity of ≥98% to those found in GenBank. A detailed phylogenetic analysis study that includes these sample is described in Manna et al. (2018).

3.5. Proposal for a laboratory work flow

A simulation to assess the additional percentage of suspect positive detected using different diagnostic workflows, was performed employing the 274 samples having the results for all of the serological and biomolecular tests (Fig. 1). In the absence of a GS, it was assumed that the positives are considered suspect of being infected. For *B. caballi*, no additional positives were detected by ELISA compared to the 86.0% identified by IFAT. Commercial PCR identified only 2% of positives

Table 1

. Values of proportion of positives to B. caballi and *T. equi* detected by immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), polymerase chain reactions (PCRs) and blood smear in the whole sample population (overall: horses and donkeys) and in the asymptomatic and suspect groups. The proportions of positive are calculated on the total number of samples analyzed by each assay, while statistical analysis was performed for each pair of assays on samples having results for both techniques. Total number of samples of each group and number of positive on the total number of analyzed samples are reported in brackets. The same superscript letter (for B. caballi) or number (for *T. equi*) within the same column indicates a statistical difference between the assays and the p-values are reported in the last row.

Assay	B. caballi			*T. equi*		
	Overall (1300)	Asymptomatic (990)	Symptomatic (310)	Overall (1300)	Asymptomatic (990)	Symptomatic (310)
IFAT	0.12^{abc} (49/416)	0.15^{abc} (35/229)	0.07^{a} (14/188)	0.42^{1} (157/371)	0.50^{1} (106/212)	0.32^{1} (51/159)
ELISA	0.05^{ade} (71/1300)	0.06^{ade} (63/990)	0.03^{ab} (8/310)	0.38^{23} (493/1300)	0.40^{23} (393/990)	0.32^{2} (100/310)
Commercial PCR	0.02^{bdf} (22/885)	0.02^{bdf} (11/574)	0.03 (11/310)	0.30^{124} (265/884)	0.34^{124} (194/575)	0.23^{12} (71/310)
Literature PCR	0.07^{cef} (40/541)	0.08^{cef} (36/435)	0.04^{b} (4/106)	0.58^{34} (315/541)	0.64^{34} (279/435)	0.34 (36/106)
Blood smear	0.07 (29/417)	0.01 (1/136)	0.10 (28/281)	0.07 (29/417)	0.01 (1/136)	0.10 (28/281)
p-value	a: <0.00001 b: <0.00001 c: <0.00001 d: <0.00001 e: 0.03572 f: <0.00001	a: <0.00001 b: <0.00001 c: 0.00038 d: <0.00001 e: 0.00932 f: <0.00001	a: 0.00634 b: 0.04338	1: <0.00001 2: <0.00001 3: 0.0002 4:<0.00001	1: 0.00008 2: <0.00001 3: <0.00001 4: <0.00001	1: 0.04338 2: 0.00906

Table 2
Values of proportion of positives to B. caballi and *T. equi* detected by immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), polymerase chain reactions (PCRs) and blood smear in the horse sample population (overall) and in the asymptomatic and suspect groups. The proportions of positive are calculated on the total number of samples analyzed by each assay, while statistical analysis was performed for each pair of assays on samples having results for both techniques. Total number of samples of each group and number of positive on the total number of analyzed samples are reported in brackets. The same superscript letter (for B. caballi) or number (for *T. equi*) indicates, within the same column, a statistical difference between the assays and the p-values are reported in the last row.

Assay	B. caballi			*T. equi*		
	Overall (993)	Asymptomatic (769)	Symptomatic (224)	Overall (993)	Asymptomatic (769)	Symptomatic (224)
IFAT	0.07^{ab} (13/196)	0 (0/37)	0.08^{ab} (13/159)	0.30 (48/161)	0 (0/30)	0.37^{1} (48/131)
ELISA	0.06^{acd} (64/993)	0.07^{ab} (58/769)	0.03^{a} (6/224)	0.34^{12} (335/993)	0.35^{12} (272/769)	0.28 (63/224)
Commercial PCR	0.02^{ce} (14/586)	0.01^{ac} (5/362)	0.04^{b} (9/224)	0.27^{13} (161/586)	0.31^{13} (114/362)	0.21^{1} (47/224)
Literature PCR	0.08^{bde} (29/363)	0.10^{bc} (26/264)	0.03 (3/99)	0.57^{23} (209/363)	0.67^{23} (177/264)	0.32 (32/99)
Blood smear	0.08 (23/292)	0 (0/95)	0.12 (23/197)	0.08 (23/292)	0 (0/95)	0.12 (23/197)
p-value	a: 0.0011 b: 0.044 c: <0.00001 d: 0.0009 e: 0.00016	a: <0.00001 b: 0.00014 c: 0.0001	a: 0.001 b: 0.044	1: <0.00001 2: <0.00001 3: <0.00001	1: <0.00001 2: <0.00001 3: <0.00001	1: 0.044

Table 3
. Values of proportion of positives to B. caballi and *T. equi* detected by immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), polymerase chain reactions (PCRs) and blood smear in the donkey sample population (overall) and in the asymptomatic and suspect groups. The proportions of positive are calculated on the total number of samples analyzed by each assay, while statistical analysis was performed for each pair of assays on samples having results for both techniques. Total number of samples of each group and number of positive on the total number of analyzed samples are reported in brackets. The same superscript letter (for B. caballi) or number (for *T. equi*) indicates, within the same column, a statistical difference between the assays and the p-values are reported in the last row.

Assay	B. caballi			*T. equi*		
	Overall (307)	Asymptomatic (221)	Symptomatic (86)	Overall (307)	Asymptomatic (221)	Symptomatic (86)
IFAT	0.16^{abc} (36/219)	0.18^{abc} (35/190)	0.05 (2/42)	0.52^{1} (109/210)	0.58^{1} (106/182)	0.11 (3/28)
ELISA	0.02^{ad} (7/306)	0.02^{ad} (5/220)	0.02 (2/86)	0.48^{2} (148/306)	0.55^{2} (121/220)	0.43^{1} (37/86)
Commercial PCR	0.03^{b} (8/298)	0.03^{b} (6/212)	0.02 (2/86)	0.35^{123} (104/298)	0.38^{123} (80/212)	0.28^{1} (24/86)
Literature PCR	0.06^{cd} (11/178)	0.06^{cd} (10/171)	0.01 (1/7)	0.59^{3} (106/178)	0.60^{3} (102/171)	0.57 (4/7)
Blood smear	0.05 (6/125)	0 (0/41)	0.06 (5/84)	0.05 (6/125)	0.02 (1/41)	0.06 (5/84)
p-value	a: <0.00001 b: <0.00001 c: 0.00038 d: 0.0111	a: <0.00001 b: <0.00001 c: 0.00038 d: 0.0188	/	1: <0.00001 2: <0.00001 3: <0.00001	1: <0.00001 2: 0.0003 3: <0.00001	1: 0.03846

Table 4
. Values of total concordance and concordance on positives, within brackets, between each pair of assays (immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), polymerase chain reactions (PCRs) and blood smears) in the whole sample population (overall: horses and donkeys) and in the asymptomatic and suspect groups. Lower half of each section is referred to B. caballi, upper half to *T. equi*.

		IFAT[a]	ELISA[b]	Commercial PCR[c]	Literature PCR[d]	Blood smear		
B. caballi	ELISA	0.86 (0.00)		0.68 (0.44)	0.71 (0.63)	0.72 (0.11)	*T. equi*	Overall
	Commercial PCR	0.87 (0.11)	0.90 (0.04)		0.75 (0.58)	0.72 (0.11)		
	Literature PCR	0.82 (0.14)	0.86 (0.14)	0.94 (0.24)		0.82 (0.48)		
	Blood smear	0.90 (0.29)	0.91 (0.03)	0.93 (0.14)	0.81 (0.10)			
	IFAT		0.84 (0.73)	0.74 (0.51)	0.80 (0.72)	1.00 (0.00)		Asymptomatic
	ELISA	0.84 (0.00)		0.62 (0.45)	0.69 (0.65)	0.81 (0.04)		
	Commercial PCR	0.86 (0.14)	0.88 (0.03)		0.71 (0.56)	0.89 (0.06)		
	Literature PCR	0.82 (0.16)	0.83 (0.14)	0.93 (0.22)		/		
	Blood smear	0.98 (0.00)	0.97 (0.00)	0.98 (0.00)	/			
	IFAT		0.71 (0.34)	0.87 (0.62)	0.85 (0.67)	0.80 (0.36)		Suspect
	ELISA	0.91 (0.00)		0.78 (0.43)	0.78 (0.44)	0.68 (0.13)		
	Commercial PCR	0.90 (0.05)	0.95 (0.12)		0.91 (0.72)	0.83 (0.30)		
	Literature PCR	0.89 (0.08)	0.96 (0.00)	0.98 (0.50)		0.82 (0.48)		
	Blood smear	0.88 (0.26)	0.88 (0.03)	0.90 (0.15)	0.81 (0.10)			

B. caballi IFA IgG Antibody Kit and B. equi IFA IgG Antibody Kit (Fuller Laboratories®).
B. caballi Antibody test kit and B. equi Antibody test kit (VMRD®, Inc, Pullman, WA, USA).
B. caballi 18S ribosomal RNA gene Standard Kit and *T. equi* Equi merozoite antigen 1 (ema-1) gene Standard Kit (Genesig®).
Primers and probes described in Bhoora et al., 2010a for B. caballi; Kim et al., 2008 for *T. equi*.

compared to literature PCR (14%) when used as first PCR on a serological negative sample. For *T. equi*, ELISA detects a percentage of positives (66.5%), that is lower than for the IFAT (83.9%), even if some positive samples (6.3%) were ELISA + /IFAT negative. Same results as for *B. caballi* were found for PCR methods, as commercial PCR when used first, detected 3.4% positives compared to the 11.2% of literature PCR positives. Blood smear was not included because only 96 samples had results for all the five assays, nevertheless all samples positives to this method (29) were confirmed as positive by at least one PCR, meaning that this assay would not detect additional positives.

PCR results of serologically positive samples are crucial to discriminate an ongoing infection from antibody persistence. In the present study, among the 71 samples positive by the *B. caballi* ELISA 4/71 (6%) was positive by commercial PCR and 12/60 (20%) positive by literature PCR, of which only one positive by both assays. Worthy of note is that all the 12 samples positive by the literature PCR belonged to asymptomatic subjects while of the four equids positive to commercial PCR, two were symptomatic and two were asymptomatic.

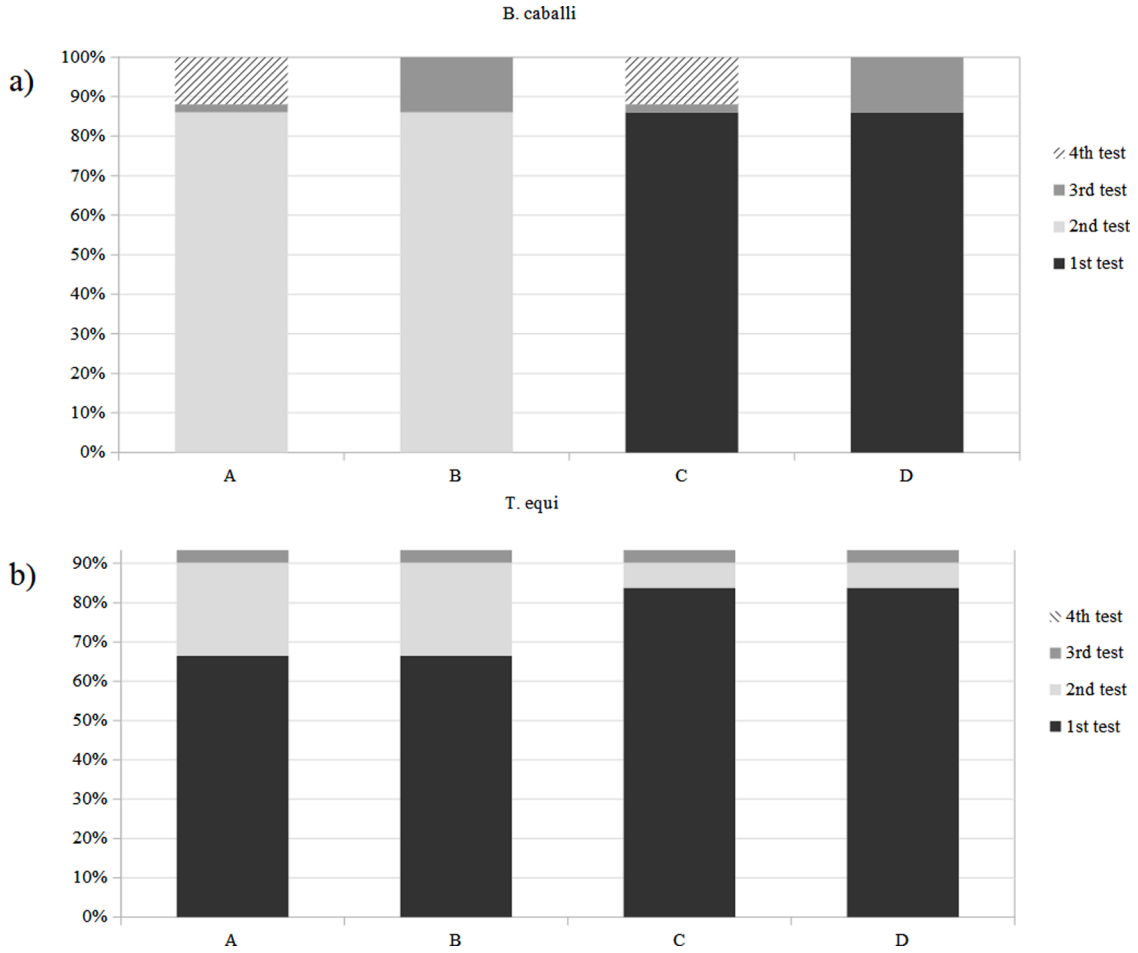

Fig. 1. . Contribution of the tests in detecting positivity to B. caballi (a) and *T. equi* (b), in terms of cumulative percentage, of two serological assays (IFAT-Fuller® and ELISA-VMRD ®) and two PCR assays (B. caballi 18S ribosomal RNA gene Standard Kit and *T. equi* Equi merozoite antigen 1 (ema-1) gene Standard Kit-Genesig ® and literature PCRs: (Kim et al., 2008, Bhoora et al., 2010a), on 274 equids samples, in four hypothetical diagnostic algorithms in which only the negative to the previous test are further analyzed with a different test. Order of use of the tests A: ELISA, IFAT, Commercial PCR, Literature PCR; B: ELISA, IFAT, Literature PCR, Commercial PCR; C: IFAT, ELISA, Commercial PCR, Literature PCR; D: IFAT, ELISA, Literature PCR, Commercial PCR.

Among the 494 *T. equi* ELISA positive, 228/480 (47.5%) resulted positive by commercial PCR, of which 177 (77.6%) were asymptomatic; and 266/374 (71%) showed positivity to the literature PCR, of which 248 (94.3%) were asymptomatic.

4. Discussion

Purpose of testing for EP is usually to declare freedom from infection of the animal or to confirm a suspect of infection or to verify efficacy of treatment and can be done through the combined use of serological and molecular methods.

A premise is necessary before discussing results compared to other studies: although some authors reported data on concordance between serological and biomolecular assays (Jaffer et al., 2010; Abedi et al., 2014; Ferreira et al., 2016; Sumbria et al., 2016a, Camino et al., 2019) is the opinion of the authors of the present study that a low concordance between the two types of assay should be expected as reported for example for by Bartolomé Del Pino et al. (2016) in Italy, or by Sumbria et al. (2016b) in India. Thus, in Table 4 concordance between serological and PCR assays are reported but not discussed. The results of both assays, instead, should be interpreted, for each subject, according to an algorithm to assess the sanitary status, as proposed in paragraph 4.3.

Commercial ELISA for *B. caballi* detected a significantly lower number of reactive samples than IFAT and with a complete disagreement on positives with the latter, in all groups of animals. These data are in contrast with Jaffer et al. (2010) who reported an agreement within positives of 0.69; but in agreement with the study of Kamyingkird et al. (2014), in which IFAT detected prevalence twice higher than ELISA with no agreement on positives Kamyingkird et al. (2014). claimed that the IFAT higher prevalence could be ascribed to non-specific reactions, but the results of the present study support the hypothesis of a lower detection capacity of the c-ELISA in view of the results obtained in comparison with the IFAT and the PCR results. These data are in agreement with Bhoora et al. (2010a), Awinda et al. (2013) and Kappmeyer et al. (1999). The latter, describing the development of the method, already reported a 6.5% of c-ELISA–/IFAT+ samples belonging to different regions of the world and for this suggested that there was no correlation between parasite strain and lack of reactivity, although not providing alternative hypothesis in the paper.

In the present study, on 376 c-ELISA negative samples, 50 (13.3%), were IFAT positive, twice as much the percentage detected by Kappmeyer et al. (1999) and, on 331 IFAT negative samples, only five were c-ELISA positive. Significant differences were detected both in asymptomatic and symptomatic groups, suggesting no correlation between the presence of symptoms and reactivity pattern. Even in case of clinical disease, the lack of historical data of the subjects did not allow a clear distinction between acute and reactivated chronic forms suggesting that maybe the different serological pattern of reactivity could be better correlated to the period between sampling and first infection. The causes of this discrepancy might be further investigated by ad hoc studies.

No statistical differences were detected between the serological assays for *T. equi* in all investigated groups, although concordance values were unsatisfactory, being 0.79 at the overall level and 0.59 for positives. Previous studies, Shkap et al. (1998), Jaffer et al. (2010) and Montes Cortés et al. (2017) reported an overall concordance values between IFAT and ELISA from 0.84 to 0.99. As in the present study the number of samples analyzed was from four to ten times higher than the other studies (1,300 compared to 316 (Shkap et al., 1998), 105 (Jaffer et al., 2010) and 108 (Montes Cortés et al., 2017)), the estimation reported in the paper is presumably more accurate, and detects a relevant number of samples that are positive only to ELISA (34) or to IFAT (45). It is worth to highlight that of the 34 ELISA positive, only five were positive to one PCR, while of 45 IFAT positive, 33 were positive to one PCR, suggesting that probably the IFAT results are better correlated to the presence of a vital parasite and that the ELISA detects declining antibodies (Wise et al., 2018). Worth of note is that IFAT, despite the fact that it may require a dilution of the sample for the elimination of an aspecific fluorescence, performed better than the ELISA supported by the evidence of being concordant with biomolecular methods. This data could be explained, as already proposed by Schelp et al. (1995) in Brazil and Morocco, with a difference in sequence between parasite strains. The phylogenetic trees for *T. equi* proposed by Manna et al. (2018), both for ema-1 and 18s, demonstrated that parasites with a wide range of genetic variability are circulating concurrently in Italy, with samples, belonging to group B of ema-1 phylogenetic tree, that are not reactive to the ELISA. Nonetheless, the ELISA for *T. equi* could still represent a valid diagnostic tool employed together with IFAT and PCR.

An accurate comparison between blood smears and serological assays cannot be discussed, as the outcome for microscopic evaluation was presence or absence of either piroplasms. However, it is worth to underline that, at a descriptive level, on two samples with a positive blood smear, positive only to PCR for *B. caballi*, none were detected as positive by the c-ELISA for *B. caballi* and only one was positive by IFAT (on the second sample the latter was not performed). Among the samples positive only to *T. equi,* IFAT detected nine out of 11, while ELISA only two out of 11. These results are counter-intuitive, as a serological test, except in case of early infections, should be more sensitive than blood smear examination (Sevinc et al., 2008). These findings further confirm a possible detection issues of serological assays, the c-ELISA in particular, even if less serious for *T. equi* than for *B. caballi*, as an important interference with efficacy in controlling EP.

Whereas the serological results seem not to show a different trend between symptomatic and asymptomatic groups, a significant difference among biomolecular assays was detected in the overall and the asymptomatic groups, in which, for both piroplasms, literature PCRs detected more positives than commercial PCRs. A substitute in the absence of a Gold Standard, is the sequencing of the PCR amplicons to confirm their specificity and, in all the samples on which it was performed and successful, the percentage of sequence identity obtained confirmed in the majority of the cases the specificity of the PCR result in which they were detected. Thus, the differences could be correlated to the different LOD of the PCRs, higher in the literature PCRs than the commercial ones, considering that in the asymptomatic group the parasitic load is expected to be more at the limit of detection. This could be more important for *B. caballi*, that infects a lower percentage of red blood cells and for which both PCRs, commercial and from literature, have the same target region even if the exact sequence for the commercial one is unknown (Bhoora et al., 2010a) *Babesia caballi*-18S rRNA gene genesig ® Advanced Kit Real Time PCR handbook HB10.03.11 Published Date: 09/11/2018).

Real-time PCRs for *T. equi* have different targets, as commercial PCR amplifies a region in the Ema-1, while the literature PCR amplifies a portion of 18S rRNA gene. The results of this study are in agreement with the study of Bhoora et al. (2010b), in which 20 samples from South Africa were negative to a PCR for Ema-1 gene and positive to PCR for 18s rRNA gene, supporting the hypothesis that sequence heterogeneity could be the reason for the discrepant results. Further confirmation studies are required also considering that the sequence amplified by the commercial kit is, as for *B. caballi,* not provided by the manufacturer that declares only "a 100% homology with a broad range of *T. equi* sequences" (*Theileria equi* Equi merozoite antigen 1 (ema-1) gene; Genesig ® Standard Kit Handbook). A recent study reports the detection of a new species of *Theileria, T. haneyi* (Knowles et al., 2018), whose pathogenic role and prevalence in Europe, together with the capability to be detected by the diagnostic methods currently in use is still to be evaluated. Moreover, performance of new PCR assays, as for example those described by Bhoora et al. (2018), Monte Cortés et al. (2019), and Lobanov et al. (2018), capable, according to the developers, to identify the prevalent agent in case of mixed infections, should be assessed and eventually employed to reduce time to response, and to enhance the identification of the parasite responsible for the clinical status. As a further comment, the higher prevalence detected by PCR in asymptomatic donkeys compared to asymptomatic horses indicates that the former species may more frequently display chronic or inapparent forms of the disease, being for this reason a threat for horses in case of coexistence of both species (Onyiche et al., 2019).

The results of this study indicate that the progress of the infection, considered from an immunologic and parasitic perspective, together with the performances of diagnostic assays can influence the accuracy of the diagnosis of EP. Presence of cross-reactions and performances issues of the several assays available should always be kept in consideration when interpreting laboratory results.

Even though c-ELISA is a prescribed test for international trade, analysing the four diagnostic pathways, it seems to have poor performancefor the serological diagnosis of *B. caballi*, at least in the Italian framework, while still helpful for *T. equi*, and for this, in case of a negative result, it should be combined with IFAT.

Considering that the different countries have not uniformly chosen the assays to be employed, and that there could be the confirmation of the laboratory result given by the exporting country, the subsequent use of the two serological tests for *T. equi* is strongly advised to identify a major number of positives and to avoid discordant results and subsequent economic losses.

Furthermore, biomolecular assays proved to be of prime importance to detect early infections that could be overlooked by a serological test. Thus, it is advisable that samples of serum and EDTA blood should be always be collected for international trade purposes, considering that a substantial time span could occur between test and movement with a possible seroconversion that would go undetected if a second sample to evaluate this is not requested.

An efficient laboratory pathway should also take into account practical aspects, considering both costs (sustained by the laboratory or by the practitioner/owner) and response time. A laboratory work flow was already described by Alanazi et al. (2014) for *T. equi* detection in blood samples. The authors propose the algorithm showed in Table 5, considering it as the most suitable, in epidemiological situations as those of Italy.

In view of the overall results, serum and EDTA blood samples should be collected irrespective of the reason (clinical suspect, international trade and control post-treatment) for which the laboratory analyzes are requested. In case of negativity for both serological and biomolecular assays the animal is considered as "free from infection".

When PCR positive, the subject is considered of harbouring an active infection if serologically positive, or suspect of having an early infection when serologically negative. In both cases further analysis are required, evaluating haemato-biochemical parameters (anaemia, renal and liver functionality), to assess the physiological status of the subject, in view of a possible pharmacological treatment. The PCR-positive/serological negative subjects should be sampled, at the least at two to three weeks interval to detect a seroconversion for the status of the animal to be assessed.

In the case of samples that are only seropositive, acute or a previous

Table 5
. Diagnostic algorithm proposed for assessing of the sanitary status for equine piroplasmosis.

		PCR		
		POSITIVE	NEGATIVE	INCONCLUSIVE
SEROLOGICAL TEST(S)[a]	POSITIVE	Active Infection: evaluate clinical and haemato-biochemical parameters for treatment. Previous/ chronic infection (no clinical signs): repeat PCR 2x template or request another sample	Active Infection (If first time positive): evaluate clinical and haemato-biochemical parameters for treatment. Previous/chronic infection, treated infections: repeat 2x template. (Immunoblot to confirm treatment efficacy)	Previous/chronic infection: repeat PCR 2x template or request another sample
	NEGATIVE	Early infections: monitor for seroconversion; evaluate clinical and haemato-biochemical parameters for treatment.	Free from infection	Suspect: repeat PCR 2x template or request another sample

a For B. caballi the serological test is only IFAT, while for *T. equi* both c-ELISA and IFAT should be employed.

or a chronic infection could be hypothesized, especially if the anamnesis reports vague and recurring symptoms, such as fatigue or a decrease in performance especially for sport horses. It is recommended that seropositive but PCR negative subjects are not to be moved and that they should be monitored for reactivation of the infection in case of stress, diseases or pharmacological treatment (corticosteroids) that could compromise the immune system, as demonstrated for other diseases, e.g. equine infectious anaemia (Autorino et al., 2016; Tumas et al., 1994) even though recently not confirmed for *T. equi* (Tirosh-Levy et al., 2020).

Other tests, as sequencing or immunoblot, should be validated according to actual OIE standards (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2018b) and employed to verify PCR and serological assays.

As for another equine disease, e.g. equine infectious anaemia, a validated IB could be a valid tool: in the case of EP it could demonstrate early infections, or verify the success of treatment, together with PCR (Scicluna et al., 2019; Wise et al., 2018).

5. Conclusion

This is the first comparison study that includes commercial and literature assays, conducted on 1,300 Italian equid samples, aiming to verify the reliability of results and assesses an efficient diagnostic algorithm of analysis. When employed in Italy, c-ELISA for *B. caballi* seems to have a poor performance, as also the commercial PCRs for both parasites. IFAT for both piroplasms and c-ELISA for *T. equi* can be useful, as well as literature PCRs for both parasites, to assess the sanitary status of a subject and address the practitioner to further analysis and to evaluate the type of pharmacological treatment.

The reasons for the general low concordance of the diagnostic methods must be investigated by analysing the gene and amino acid sequences, as the results could suggest divergences between the strains circulating worldwide. An improvement and verification of c-ELISAs validation should be considered by the manufacturers, and, at the current state of knowledge, the first employment in countries with no historical serosurvey data available should be conducted carefully.

This study strongly reinforces the evidence that methods employed in a specific region should be developed employing antigens and sequences detected locally, even if this could be difficult to achieve.

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CRediT authorship contribution statement

Roberto Nardini: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Antonella Cersini:** Methodology, Investigation, Writing – review & editing. **Leticia Elisa Bartolomé Del Pino:** Methodology, Investigation. **Giuseppe Manna:** Methodology, Investigation, Writing – review & editing. **Manuela Scarpulla:** Investigation, Writing – review & editing. **Alessandra Di Egidio:** . **Roberta Giordani:** . **Valeria Antognetti:** . **Vincenzo Veneziano:** . **Maria Teresa Scicluna:** Supervision, Project administration, Funding acquisition, Conceptualization, Methodology, Validation, Writing – review & editing.

Declaration of Competing Interest

None

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.101939.

References

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

Ahmed, A., Engelberts, M.F.M., Boer, K.R., Ahmed, N., Hartskeerl, R.A., 2009. Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. PLoS One 4, 4–11. https://doi.org/10.1371/journal.pone.0007093.

Alanazi, A.D., Said, A.E., Morin-adeline, V., Alyousif, M.S., Šlapeta, J., Slapeta, J., 2014. Quantitative PCR detection of *Theileria equi* using laboratory workflows to detect asymptomatic persistently infected horses. Vet. Parasitol. 206, 138–145. https://doi.org/10.1016/j.vetpar.2014.09.019.

Autorino, G.L., Eleni, C., Manna, G., Frontoso, R., Nardini, R., Cocumelli, C., Rosone, F., Caprioli, A., Alfieri, L., Scicluna, M.T., 2016. Evolution of equine infectious anaemia in naturally infected mules with different serological reactivity patterns prior and after immune suppression. Vet. Microbiol. 189 https://doi.org/10.1016/j.vetmic.2016.04.003.

Awinda, P.O., Mealey, R.H., Williams, L.B.A., Conrad, P.A., Packham, A.E., Reif, K.E., Grause, J.F., Pelzel-McCluskey, A.M., Chung, C., Bastos, R.G., Kappmeyer, L.S., Howe, D.K., Ness, S.L., Knowles, D.P., Ueti, M.W., 2013. Serum antibodies from a subset of horses positive for *Babesia caballi* by competitive enzyme-linked immunosorbent assay demonstrate a protein recognition pattern that is not consistent with infection. Clin. Vaccine Immunol. 20, 1752–1757. https://doi.org/10.1128/CVI.00479-13.

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

Battsetseg, B., Xuan, X., Ikadai, H., Bautista, J.L.R., Byambaa, B., Boldbaatar, D., Battur, B., Battsetseg, G., Batsukh, Z., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2001. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. Int. J. Parasitol. 31, 384–386. https://doi.org/10.1016/S0020-7519(01)00120-5.

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 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. https://doi.org/10.1016/j.vetpar.2009.11.011.

Bhoora, R., Quan, M., Matjila, P.T., Zweygarth, E., Guthrie, A.J., Collins, N.E., 2010b. 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.

Bhoora, R.V., Pienaar, R., Cornelius, F., Josemans, A., Matthee, O., Marumo, R., Troskie, C., Mans, B.J., 2018. Multiplex hydrolysis-probe assay for the simultaneous detection of *Theileria equi* and *Babesia caballi* infections in equids. Vet. Parasitol. 255, 61–68. https://doi.org/10.1016/j.vetpar.2018.03.022.

Brüning, A., 1996. Equine piroplasmosis an update on diagnosis, treatment and prevention. Br. Vet. J. 152 (2), 139–151. https://doi.org/10.1016/S0007-1935(96)80070-4.

Camino, E., Dorrego, A., Carvajal, K.A., Buendia-Andres, A., de Juan, L., Dominguez, L., Cruz-Lopez, F., 2019. Serological, molecular and hematological diagnosis in horses with clinical suspicion of equine piroplasmosis: pooling strengths. Vet. Parasitol. 275, 108928 https://doi.org/10.1016/j.vetpar.2019.108928.

Council Directive 2009/156/EC of 30 November 2009 on animal health conditions governing the movement and importation from third countries of equidae. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32009L0156.

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.

Faine, S., Adler, B., Bolin, C., Perolat, P., 1999. *Leptospira* and Leptospirosis. Medisci, Melbourne.

Ferreira, E.P., Vidotto, O., Almeida, J.C., Ribeiro, L.P.S., Borges, M.V., Pequeno, W.H.C., Stipp, D.T., de Oliveira, C.J.B., Biondo, A.W., Vieira, T.S.W.J., Vieira, R.F.C., 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.

Friedhoff, K.T., Tenter, A.M., Müller, I., 1990. Haemoparasites of equines: impact on international trade of horses. Rev. Sci. Tech. 9, 1187–1194.

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. https://doi.org/10.1016/j.tvjl.2012.10.025.

Hirato, K., Ninomiya, N., Uwano, Y., Kutil, T., 1945. Studies on the complement fixation reaction for equine piroplasmosis. Jpn. J. Vet. Sci. 7, 204–205.

Jaffer, O., Abdishakur, F., Schuster, R.K., 2010. A comparative study of serological tests and PCR for the diagnosis of equine piroplasmosis. Parasitol. Res. 106, 709–713. https://doi.org/10.1007/s00436-009-1669-5.

Kamyingkird, K., Yangtara, S., Desquesnes, M., Cao, S., Adjou Moumouni, P.K., Jittapalapong, S., Nimsupan, B., Terkawi, M.A., Masatani, T., Nishikawa, Y., Igarashi, I., Igarashi, I., Xuan, X., 2014. Seroprevalence of *Babesia caballi* and *Theileria equi* in horses and mules from Northern Thailand. J. Protozool. Res. 24, 11–17. https://doi.org/10.32268/jprotozoolres.24.1-2_11.

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. https://doi.org/10.1128/AEM.67.4.1893.

Kim, C., Blanco, L.B.C., 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.

Knowles, D.P., Kappmeyer, L.S., Haney, D., Herndon, D.R., Fry, L.M., Munro, J.B., Sears, K., Ueti, M.W., Wise, L.N., Silva, M., Schneider, D.A., Grause, J., White, S.N., Tretina, K., Bishop, R.P., Odongo, D.O., Pelzel-McCluskey, A.M., Scoles, G.A., Mealey, R.H., Silva, J.C., 2018. Discovery of a novel species, *Theileria haneyi* n. sp., infective to equids, highlights exceptional genomic diversity within the genus *Theileria*: implications for apicomplexan parasite surveillance. Int. J. Parasitol. 48, 679–690. https://doi.org/10.1016/j.ijpara.2018.03.010.

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.

Lobanov, V.A., Peckle, M., Massard, C.L., Brad Scandrett, W., Gajadhar, A.A., 2018. Development and validation of a duplex real-time PCR assay for the diagnosis of equine piroplasmosis. Parasites Vectors 11, 1–12. https://doi.org/10.1186/s13071-018-2751-6.

Madden, P.A., Holbrook, A.A., 1968. Equine piroplasmosis: indirect fluorescent antibody test for *Babesia caballi*. Am. J. Vet. Res. 29, 117–123.

Manna, G., Cersini, A., Nardini, R., Bartolomé Del Pino, L.E., Antognetti, V., Zini, M., Conti, R., Lorenzetti, R., Veneziano, V., Autorino, G.L., Scicluna, M.T., 2018. Genetic diversity of *Theileria equi* and *Babesia caballi* infecting horses of Central-Southern Italy and preliminary results of its correlation with clinical and serological status. Ticks Tick Borne Dis. 9, 1212–1220. https://doi.org/10.1016/j.ttbdis.2018.05.005.

Massung, R.F., Slater, K., Owens, J.H., Nicholson, W.L., Mather, T.N., Solberg, V.B., Olson, J.G., 1998. Nested PCR assay for detection of granulocytic ehrlichiae. J. Clin. Microbiol. 36, 1090–1095. https://doi.org/10.1128/jcm.36.4.1090-1095.1998.

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

Montes-Cortés, M.G., Fernandez-Garcia, J.L., Martínez-Estéllez, M.Á.H., 2019. A multinested PCR for detection of the equine piroplasmids *Babesia caballi* and *Theileria equi*. Ticks Tick Borne Dis. 10, 305–313. https://doi.org/10.1016/j.ttbdis.2018.11.008.

Nagore, D., García-Sanmartín, J., García-Pérez, A.L., Juste, R.A., Hurtado, A., 2004. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: Epidemiological survey and phylogenetic analysis. Vet. Parasitol. 123, 41–54. https://doi.org/10.1016/j.vetpar.2004.04.010.

Nardini, R., Autorino, G.L., Ricci, I., Frontoso, R., Rosone, F., Simula, M., Scicluna, M.T., 2016. Validation according to OIE criteria of a monoclonal, recombinant p26–based, serologic competitive enzyme-linked immunosorbent assay as screening method in surveillance programs for the detection of equine infectious anemia virus antibodies. J. Vet. Diagn. Investig. 28, 88–97. https://doi.org/10.1177/1040638715625092.

Nardini, R., Bartolomé Del Pino, L.E., Cersini, A., Manna, G., Viola, M.R., Antognetti, V., Autorino, G.L., Scicluna, M.T., 2021. Comparison of PCR-based methods for the detection of *Babesia caballi* and *Theileria equi* in field samples collected in Central Italy. Parasitol. Res. 120, 2157–2164. https://doi.org/10.1007/s00436-021-07153-4.

Nicolaiewsky, T.B.B., Richter, M.F.F., Lunge, V.R.R., Cunha, C.W.W., Delagostin, O., Ikuta, N., Fonseca, A.S., Da Silva, S.S., Ozaki, L.S., 2001. Detection of *Babesia equi* (Laveran, 1901) by nested polymerase chain reaction. Vet. Parasitol. 101, 9–21. https://doi.org/10.1016/S0304-4017(01)00471-X.

OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2018a (Web version) Chapter 3.5.8. Equine piroplasmosis (Version adopted in May 2014) http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.05.08_EQUINE_PIROPLASMOSIS.pdf.

OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2018b (Web version) Chapter 2.2.1. Development and optimisation of antibody detection assays (Version adopted in May 2014) http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.02.01_ANTIBODY_DETECT.pdf.

Onyiche, T.E., Suganuma, K., Igarashi, I., Yokoyama, N., Xuan, X., Thekisoe, O., 2019. A review on equine piroplasmosis: epidemiology, vector ecology, risk factors, host immunity, diagnosis and control. Int. J. Environ. Res. Public Health 16, 1736. https://doi.org/10.3390/ijerph16101736.

Ristic, M., Sibinovic, S., 1964. Equine babesiosis: diagnosis by a precipitation in gel and by a one-step fluorescent antibody-inhibition test. Am. J. Vet. Res. 25, 1519–1526.

Rothschild, C.M., 2013. Equine piroplasmosis. J. Equine Vet. Sci. 33, 497–508. https://doi.org/10.1016/j.jevs.2013.03.189.

Rothschild, C., Knowles, D., Sellon, D.C., Long, M., 2007. Equine piroplasmosis (eds.). Equine Infectious Diseases. Elsevier, USA, pp. 465–473.

Schelp, C., Böse, R., Micha, A., Hentrich, B., 1995. Cloning and expression of two genes from *Babesia equi* merozoites and evaluation of their diagnostic potential. Appl. Parasitol. 36, 1–10.

Schnittger, L., Rodriguez, A.E., Florin-Christensen, M., Morrison, D.A., 2012. *Babesia*: a world emerging. Infect. Genet. Evol. 12, 1788–1809. https://doi.org/10.1016/j.meegid.2012.07.004.

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. https://doi.org/10.1128/AAC.00404-09.

Scicluna, M.T., Autorino, G.L., Cook, S.J., Issel, C.J., Cook, R.F., Nardini, R., 2019. Validation of an immunoblot assay employing an objective reading system and used as a confirmatory test in equine infectious anaemia surveillance programs. J. Virol. Methods 266, 77–88. https://doi.org/10.1016/j.jviromet.2019.01.012.

Scoles, G.A., Ueti, M.W., 2015. Vector ecology of equine piroplasmosis. Annu. Rev. Entomol. 561–580. https://doi.org/10.1146/annurev-ento-010814-021110.

Sevinc, F., Maden, M., Kumas, C., Sevinc, M., Ekici, O.D., 2008. A comparative study on the prevalence of *Theileria equi* and *Babesia caballi* infections in horse sub-populations in Turkey. Vet. Parasitol. 156, 173–177. https://doi.org/10.1016/j.vetpar.2008.06.006.

Shkap, V., Cohen, I., Leibovitz, B., Pipano, E., Avni, G., Shofer, S., Giger, U., Kappmeyer, L., Knowles, D., Savitsky, 1998. Seroprevalence of *Babesia equi* among horses in Israel using competitive inhibition ELISA and IFA assays. Vet. Parasitol. 76, 251–259. https://doi.org/10.1016/S0304-4017(97)00228-8.

Sumbria, D., Moudgil, A.D., Das Singla, L., 2014. Equine piroplasmosis: current status. Veterinaria 2, 9–14.

Sumbria, D., Singla, L.D., Sharma, A., Bal, M.S., Kumar, S., 2015. Multiplex PCR for detection of *Trypanosoma evansi* and *Theileria equi* in equids of Punjab, India. Vet. Parasitol. 211, 293–299. https://doi.org/10.1016/j.vetpar.2015.05.018.

Sumbria, D., Singla, L.D., 2015. Recent diagnostic and control approaches in equine piroplasmosis. Veterinaria 2, 1–6.

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

Sumbria, D., Singla, L.D., Kumar, S., Sharma, A., Dahiya, R.K., Setia, R., 2016b. Spatial distribution, risk factors and haemato-biochemical alterations associated with *Theileria equi* infected equids of Punjab (India) diagnosed by indirect ELISA and nested PCR. Acta Trop. 155, 104–112. https://doi.org/10.1016/j.actatropica.2015.12.020.

Sumbria, D., Singla, L.D., Kumar, R., Bal, M.S., Kaur, P., 2017. Comparative sero-prevalence and risk factor analysis of *Trypanosoma evansi* infection in equines from different agro-climatic zones of Punjab (India). Rev. Sci. Tech. 36, 971–979. https://doi.org/10.20506/rst.36.3.2729.

Tirosh-Levy, S., Gottlieb, Y., Steinman, A., 2020. Stress conditions do not affect *Theileria equi* parasitemia levels in sub-clinically infected horses. Ticks Tick Borne Dis. 11, 101384 https://doi.org/10.1016/j.ttbdis.2020.101384.

Tumas, D.B., Hines, M.T., Perryman, L.E., Davis, W.C., McGuire, T.C., 1994. Corticosteroid immunosuppression and monoclonal antibody-mediated CD5+ T lymphocyte depletion in normal and equine infectious anaemia virus-carrier horses. J. Gen. Virol. 75, 959–968. https://doi.org/10.1099/0022-1317-75-5-959.

Ueti, M.W., Mealey, R.H., Kappmeyer, L.S., White, S.N., Kumpula-McWhirter, N., Pelzel, A.M., Grause, J.F., Bunn, T.O., Schwartz, A., Traub-Dargatz, J.L., Hendrickson, A., Espy, B., Guthrie, A.J., Fowler, W.K., Knowles, D.P., 2012. Re-emergence of the apicomplexan *Theileria equi* in the United States: elimination of

persistent infection and transmission risk. PLoS One 7, 5–10. https://doi.org/10.1371/journal.pone.0044713.

Wise, L.N., Kappmeyer, L.S., Silva, M.G., White, S.N., Grause, J.F., Knowles, D.P., 2018. Verification of post-chemotherapeutic clearance of *Theileria equi* through concordance of nested PCR and immunoblot. Ticks Tick Borne Dis. 9, 135–140. https://doi.org/10.1016/j.ttbdis.2017.08.007.

Wise, L.N., Pelzel-McCluskey, A.M., Mealey, R.H., Knowles, D.P., 2014. Equine piroplasmosis. Vet. Clin. N. Am. Equine Pract. 30, 677–693. https://doi.org/10.1016/j.cveq.2014.08.008.