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1 **Horses infected by Piroplasms different from *Babesia caballi* and**
2 ***Theileria equi*: species identification and risk factors analysis in Italy**

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16 **Abstract**

17 Equine Piroplasmosis (EP) caused by *Theileria equi* and *Babesia caballi* is a disease affecting the
18 health and the international movement of horses. In order to assess prevalence of Piroplasmid
19 infection in the Northwestern part of Italy and to evaluate the associated risk factors, whole blood
20 was collected from 135 horses from 7 different stables across the study area. PCR and sequencing
21 were used to assess prevalence of infection and to identify detected Piroplasms to species level. A
22 total of 23 horses (P=17.04%; CI95%: 10.70-23.38%) was found to be infected with Piroplasms and
23 *T. equi* was the most prevalent species, found in 18 animals (P=13.33%; CI95%: 7.60%-19.07%).
24 Although *B. caballi* was never detected, the presence of parasites belonging to the genus *Babesia*
25 was confirmed by sequencing in 5 horses, 3 of which were infected with *B. canis* (P=2.22%; CI95%
26 0.76%-6.33%), and 2 with *B. capreoli* (P=1.48%; CI95% 0.41%-5.24%). The natural reservoir hosts of
27 *B. canis* and *B. capreoli* are the domestic dog and roe deer *Capreolus capreolus* respectively. These
28 findings pose attention to the need of considering in future epidemiological and clinical studies,
29 other Apicomplexan species as able to infect horses.

30 **Key words:** Equine Piroplasmosis, *Theileria equi*, *Babesia caballi*, *Babesia canis*, *Babesia capreoli*,
31 Italy

32 **Introduction:**

33 Equine piroplasmosis (EP) is a disease that affects Equids, caused by Apicomplexan parasites
34 *Theileria equi* and *Babesia caballi*. In endemic areas, infection occurs with varying degrees of
35 severity from subclinical to life-threatening (Guidi et al., 2014). In Italy *Ixodes ricinus*, *Dermacentor*
36 *marginatus* (Iori et al., 2010), *Rhipicephalus sanguineus*, *Rhipicephalus annulatus*, *Rhipicephalus*
37 *bursa* (Scoles and Ueti, 2015) are the competent vectors of *T. equi* and *B. caballi*. *I. ricinus* has a
38 telotropic behavior with a host range that includes many species of birds and small to large
39 mammals, including horses. In Italy, the abundance of *I. ricinus* and the probability of acquiring *I.*

40 *ricinus*-vectored diseases have been directly related to the abundance of Roe deer *Capreolus*
41 *capreolus* (Rizzoli et al., 2009). Roe deer which is the main reservoir host of *Babesia capreoli*
42 (Maladrin et al., 2010) has been expanding its presence to new areas and it increased in number
43 throughout its entire presence area in Northern Italy (Carnevali et al., 2009) which led to a
44 concomitant expansion of tick presence area and abundance. (Vor et al., 2010). *R. sanguineus* has
45 the domestic dog as main host, but it can be found on a range of wild and domestic animals,
46 horses included (Salman and Tarres-Call, 2013). In the study area *B. capreoli* is the most prevalent
47 Piroplasmid species, infecting 43.46% of free-ranging Roe deer, Red deer and Alpine chamois
48 (Zanet et al., 2014). Canine Piroplasmosis is also endemic in Italy as in most European countries, *B.*
49 *canis* was molecularly identified in 2.3% of asymptomatic dogs (Cassini et al., 2009). Host-tick-
50 pathogen relationship undergoes constant changes, mainly due to environmental, climatic and
51 anthropogenic alterations. Recently, a horse with suspected EP was found infected with *Babesia*
52 *canis* (Criado-Fornelio et al., 2003), while *Theileria annae*, *Theileria sergenti* and *Theileria buffeli*
53 were isolated from horses in Italy (Moretti et al., 2010). In this context, even the epidemiology of
54 the long known EP needs to be re-evaluated and the goal of this work is indeed to evaluate the
55 prevalence of Piroplasms infection in horses from Northwestern Italy, to identify the species of
56 Piroplasms involved and individual and environmental factors that might influence their infective
57 ability.

58 **Materials and methods:**

59 Seven stables were randomly selected in the Piedmont Region, Northwestern Italy. Whole blood
60 was collected from 135 horses and stored at -20°C until further analysis. Individuals younger than
61 2 years were not included in the study. Total genomic DNA was extracted using PureLink Genomic
62 DNA Mini Kit (Invitrogen, USA) following manufacturer's instructions. Direct molecular detection
63 of *Babesia* spp./*Theileria* spp. DNA was carried out on all samples using a semi-nested PCR

64 protocol targeting the V4 hyper-variable region of the 18S rDNA as specified elsewhere (Zanet et
65 al., 2014). In specimens where the detection of unexpected Piroplasmid species would be
66 considered as a possible mistake in sample handling, we used specific PCR protocols, as reported
67 by their respective authors, to confirm the presence of horse DNA (primers ATPase8 and ATPase6;
68 Kesmen et al., 2007) and to exclude the presence of roe deer (primers 12SCC-FW and 12SCERV-
69 REV; Fajardo et al., 2007) and dog DNA (primers CAN-F and CAN-R; Criado-Fornelio et al., 2003). To
70 confirm Piroplasmid species identification, the entire 18S rRNA gene was amplified as reported by
71 Maladrin et al. (2010). Positive amplicons were purified using QIAQuick PCR purification kit
72 (QIAGEN) and directly sequenced on both DNA strands (Macrogen, The Netherlands). The
73 resulting sequences were compared with homologous sequences available in Genbank using the
74 Basic Local Alignment Search Tool (BLAST). Multiple sequence alignments were constructed using
75 the Clustal W algorithm and a Maximum Likelihood (ML) phylogenetic analysis was performed in
76 MEGA 6 using the Kimura 2-parameter model with Gamma distributed rates (Tamura et al., 2013).
77 Accuracy of inferred topologies were assessed via bootstrap analysis.

78 For each horse, a questionnaire was used to collect information on individual and environmental
79 factors that might influence their exposure to Apicomplexan parasites. The information included in
80 the questionnaire were: breed (Italian Saddle, Trotter, English Pureblood, mixed breed), age class,
81 (2-10 years, 11-20 years, >20 years), gender, activity and movements, treatment against
82 ectoparasites, recovering of ticks, frequency and type of deworming, province of origin, as well as
83 type of housing (box, paddock, both box and paddock). To identify variables associated with
84 *Babesia/Theileria* sp. infection we used generalized linear mixed models with PCR result was the
85 dichotomous response variable and stable of origin as random effect. Also we derived from a
86 Geographic Information System (GIS) the area occupied by pastures, forest, urbanized and
87 agricultural activities within 5 km from the sampled stable (area of influence of management

88 activities of the stable). The normalized area of each land-use class was included in the model.
89 Variance inflation Factor (VIF) was used to test and avoid multicollinearity among predictors (Zuur
90 et al., 2009). Best model selection was performed using AIC (Akaike information Criterion), while
91 the goodness-of-fit of the final model was assessed by computing the area under the curve (AUC)
92 of the receiver operating characteristic plots.

93 **Results:**

94 Among the 135 horses included in the study, 23 tested positive to semi-nested PCR (P=17.04%,
95 IC95% 10.70-23.38). Among these, 18 horses (P=13.33%, IC95%7.60-19.07) were found to be
96 infected with *T. equi*. *Babesia* sp. infection was detected in the remaining 5 positive horses (P=
97 3.70%, IC95% 0.5-6.89). Detailed prevalence data are summarized in Table 1.

98 Sequencing of the entire 18S rRNA gene allowed to identify the *Babesia* isolates as belonging to *B.*
99 *canis* (n=3, P=2.22%, IC95% 0.76-6.33; GenBank accession numbers: KX839230- KX839232) and to
100 *Babesia capreoli* (n=2, P=1.48%, IC95% 0.41-5.24; GenBank accession numbers: KX839233
101 KX839234). Statistical support to species identification was given through ML phylogenetic analysis
102 (Fig.1). The PCR protocols used to confirm the presence of horse DNA (all samples tested positive)
103 and the absence of dog/roe deer DNA (all samples tested negative) excluded the possibility of a
104 wrong diagnosis due to mishandling of the samples. The presence of *T. equi* DNA was detected
105 and confirmed by sequencing in 18 horses.

106 The risk factor analysis was carried out on the basis of data collected using the questionnaire and
107 on data deriving from the GIS analysis. The covariates retained by VIF analysis and therefore used
108 to train the model were: sex, age class, housing, activity, movements, province of origin and the
109 GIS environmental parameters. The covariates included in the model with the highest predicting
110 capability (AUC=0.76) are: activity (daily recreational walks in forests and bush) which was
111 positively associated to higher risk of infection (p<0.01; OR= 2.3, IC95%= 1.2-3.4), as well as age

112 (animals from 2 to 10 years resulting more affected by Piroplasmid infection, $p < 0.05$; OR= 1.9,
113 IC95%= 1.2-2.5). Regular deworming was instead negatively associated to infection ($p < 0.05$;
114 OR=0.25, IC95%= 0.1-0.75).

115 **Discussion:**

116 EP in horses is traditionally associated to infection with *T. equi* and with *B. caballi*. *T. equi* is
117 worldwide more prevalent than *B. caballi*. (Bruning, 1996; Friedhoff et al., 1990). In Italy, *T. equi*
118 prevalence of infection ranges from 8.2% (Grandi et al., 2011) to 82.8% (Zobba et al., 2008), while
119 *B. caballi* reaches 31.5% (Torina et al., 2007). Our results fall within the range of infection already
120 reported for the country, even though to our knowledge, this is the first study dealing with horses
121 in Northwestern Italy. The most prevalent Piroplasm was *T. equi* while no *B. caballi* was detected
122 in any of the sampled horses. Atypical *Babesia* species: *B. canis* and *B. capreoli* were detected with
123 a prevalence of 3.70%. These species have been associated to infection in the domestic dog and
124 Roe deer respectively (Criado Fornelio et al., 2003; Zanet et al., 2014). Two of the 3 isolates of *B.*
125 *canis* detected in the current study, are identical to each other (GenBank Accession n.: **KX839230**,
126 **KX839231**) and match perfectly with the *B. canis* (GenBank Accession n.: **AY150060**) detected by
127 Criado Fornelio et al. (2003) in a horse with suspected EP and with isolates deposited in GenBank
128 (Accession numbers: **EU165369**, **AY962187**, **AY703073**, **AY321119**, **KC902833**, **KC593879**) which
129 were isolated from dogs, foxes or *R. sanguineus* ticks from Continental Europe (Poland, The
130 Netherlands, France) and Siberia. For what concerns *B. capreoli*, the two positive horses were
131 found infected with identical (100% identity, 100% query coverage) strains, that also perfectly
132 matched the parasites isolated in the same area, from roe deer (GenBank Accession n.: **KF773723**)
133 and red deer (GenBank Accession n.: **KF773718**) (Zanet et al., 2014). Risk factor analysis evidenced
134 how young animals (from 2 to 10 years old) and recreational horses that are ridden in the
135 countryside, are more exposed to infection with *Theileria/Babesia*. Higher rates of infections in

136 young animals are a common finding in endemic areas, where animals are exposed to EP causal
137 agents at young age and eventually clear the infection in the following years (Scoles and Ueti,
138 2015; Farkas et al.,2013). As previously reported (Peckle et al., 2013), our data evidence how horse
139 activities that increase the exposure of animals to infected ticks enhance the risk of infection. In
140 the present study recreational horses were significantly more exposed to Piroplasms infection
141 than racing/breeding horses. Regular deworming was identified as protective factor, as horses that
142 are regularly dewormed have statistically lower prevalence of infection. As confirmed by other
143 studies (Guidi et al., 2014, Moretti et al. 2010; Santos et al.2011; Vieira et al. in 2013) deworming
144 like vaccination, and flies control, are indicators of good herd management practices and may
145 reflect a reduced exposure to tick infestation and thus to a lower risk of infection. Among the risk
146 factors that were taken into account none of the geographic covariates were able to efficiently
147 discriminate the risk of infection. The number of horses infected with *B. canis* or *B. capreoli* was
148 not sufficient to make inference on significant risk factors specific for these atypical *Babesia*
149 species. **Conclusions**

150 EP is a disease of clinical and economical relevance worldwide. Despite the efforts made to control
151 the geographic expansion of the disease, the epidemiology of Piroplasms infection is constantly
152 evolving. The results of this work evidence how horses can be infected with Piroplasms species
153 different from *T. equi* and *B. caballi*. Piroplasmosis, as well as numerous vector-borne diseases
154 have been experiencing both a territorial expansion and drastic changes in host-vector
155 relationship. Economic, social and bioclimatic changes are causing an always increasing contact
156 among wildlife, humans and domestic animals (Daszak et al., 2001) and the role of wildlife as
157 source of diseases is to be specially monitored (Daszak et al., 2000). Further effort is required to
158 understand the clinical impact of atypical *Babesia* infections in horses, but clinicians, official
159 veterinary authorities and research institutions should account for the possibility of horses being

160 infected with species of Piroplasms different from *B. caballi* and *T. equi* and should adapt
161 diagnostic tools accordingly.

162 **Authors' contributions**

163 SZ performed the statistical analysis, molecular testing and prepared the manuscript, BM collected
164 blood samples, administered the questionnaires and performed analysis, AT performed the
165 molecular analysis, IT collected blood samples and connected to owners of the horses, EF designed
166 and coordinated the study. All authors read and approved the final version of the manuscript.

167 **Conflict of interests statement**

168 The authors declare that they have no competing interests.

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249 **Captions to figure:**

250 Fig. 1 Molecular Phylogenetic analysis by Maximum Likelihood method based on the Kimura 2-
251 parameter model of 18S rRNA gene of 15 *Babesia* spp. and *Theileria* spp. sequences. Isolates from
252 this study are indicated with a black arrow.