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

Zanet Stefania[1], Bassano Marina[1], Trisciuoglio Anna[2], Taricco Ivo[1], Ferroglio Ezio[1]

[1]*University of Turin, Dept. of Veterinary Sciences, Largo Braccini 2, 10095 Grugliasco (To), Italy.*

[2]*University of Turin, Dept. of Agricultural, Forest and Food Sciences, Largo Braccini 2, 10095 Grugliasco (To), Italy.*

Corresponding author:

Ezio Ferroglio

Largo Braccini 2, 10095 Grugliasco (Torino), Italy

Email: ezio.ferroglio@unito.it

Telephone: +390116709002

Abstract

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

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

Introduction:

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

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

Materials and methods:

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

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

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

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

Results:

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

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

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

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

Discussion:

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

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

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

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

Authors' contributions

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

Conflict of interests statement

The authors declare that they have no competing interests.

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Captions to figure:

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