

## Prevalence of Tick Borne Pathogens in Horses from Italy

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**ABSTRACT.** In order to investigate the prevalence of tick-borne diseases, equine piroplasmosis, equine granulocytic anaplasmosis and Lyme borreliosis in Central Italy, blood samples from 300 horses were analyzed for the presence of antibodies against *Babesia caballi*, *Theileria equi*, *Anaplasma phagocytophilum* and *Borrelia burgdorferi* using the IFAT. The blood samples were also subjected to PCR assays in order to detect pathogen DNA. A total of 78 (26.0%) and 123 (41.0%) horses were found to be seropositive for *B. caballi* and *T. equi*, respectively, while 41 (13.4%) and 21 (7.0%) horses were, respectively, seropositive for *A. phagocytophilum* and *B. burgdorferi*. Seropositivity for more than one agent was detected in 76 horses using IFAT. The most common association observed was between *T. equi* and *B. caballi* (14.7%). In addition, 54 horses (18.0%) were found to be positive for one or more tick-borne pathogens (TBPs) using PCR testing. Among these, 28 (9.3%) harbored single infections, while 26 (8.7%) were found to be co-infected with two or more pathogens. The correlation (K value) between IFAT and PCR results was 0.32 for *T. equi*, 0.34 for *B. caballi*, 0.62 for *B. burgdorferi* and 0.48 for *A. phagocytophilum*, reflecting an unprecedented degree of multiple exposures to TBPs in horses.

**KEY WORDS:** equine granulocytic anaplasmosis, equine piroplasmosis, horse, Italy, Lyme borreliosis.

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Ticks, along with mosquitoes, are considered to be the main arthropod vectors of disease agents in humans and domestic animals and, in recent years, a worldwide increase of tick-borne diseases (TBDs) has been observed [13]. Climatic and socio-economic changes influencing their ecology, in association with their high biological plasticity, have allowed ticks to extend their range of hosts and their spatial and temporal distribution. As a result, the lengthening of the seasonal transmission of tick borne pathogens (TBPs) has been observed [37].

Due to their frequent outdoor activity, horses carry a high risk of exposure to adult tick bites [6] and consequently, of infection with TBPs; in addition, the geographical redistribution of infected horses and ticks, both within and in between nations, may play a role in the spread of these diseases through equine populations around the world.

The epidemiology of TBDs in horses has been described extensively in several countries [3, 7, 9, 21, 42]. In Italy, however, reports have been relatively limited, with the majority regarding the agents of equine piroplasmosis (EP), *Theileria equi* and *Babesia caballi* [3, 18, 19, 26, 27, 39].

Equine piroplasmosis is endemic in most equine populations in tropical and subtropical areas of the world and may be associated with clinical signs including fever, ventral edema, icteric sclera, pale mucous membranes, dark urine, anemia, weakness, lethargy, reduced feed intake and mild colic with reduced fecal output [30].

Other blood-borne pathogens, namely, *Anaplasma phagocytophilum*, the agent of equine granulocytic anaplasmosis (EGA) and *Borrelia burgdorferi* sensu lato (s.l.), the causative agent of Lyme borreliosis (LB), have also been subjected to limited epidemiological studies in Italy [16, 18, 28, 40]. These bacteria infect horses in all parts of the world where *Ixodes* spp., the main tick vectors, are present [6, 41]. While most of these infections remain asymptomatic, approximately 5–10% of infected animals are thought to develop clinical presentations, which may vary significantly [20]. *A. phagocytophilum* infecting equine neutrophils and eosinophils, can cause a wide range of clinical signs including lethargy, depression, fever, limb edema, petechiation, ataxia and thrombocytopenia [5]. Clinical signs of Lyme borreliosis in horses are nonspecific and may include chronic weight loss, low grade fever, sporadic lameness, swollen joints and anterior uveitis [29].

In addition to being agents of animal disease, *A. phagocytophilum* and *B. burgdorferi* can also infect humans [11, 15], and therefore, both their zoonotic potential and the epidemiological role of the horse need to be considered. Even though horses are often described as accidental hosts for these pathogens, due to the lack of high and persistent

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bacteremia [6, 21], it appears that equine hosts may actually play an important indirect role in the epidemiological cycle of both *B. burgdorferi* and *A. phagocytophilum* infections. In fact, horses represent a good source of nutrients for the different stages of *Ixodidae* vectors, favoring their proliferation and dissemination in the environment along with the pathogens that they harbor. Furthermore, because of the persistent association with vectors, the regular sharing of habitat with people and the strong and specific immune response developed against both *B. burgdorferi* and *A. phagocytophilum*, it is, therefore, possible that equine hosts may actually represent a preferred vehicle of infection and a valid model for the evaluation of the zoonotic risk due to these pathogens.

The present study is aimed to conduct a cross-sectional survey to analyze the presence and prevalence of *B. caballi*, *T. equi*, *A. phagocytophilum* and *B. burgdorferi* infections in indigenous horse populations reared in different areas of Central Italy by using serological and molecular methods.

## MATERIALS AND METHODS

**Sample collection and laboratory analysis:** Between February 2010 and September 2011, 300 horses of different age and gender were selected to participate to the cross-sectional survey. The animals originated from farms of variable nature and size, including horseback riding schools and stud farms located in 3 regions of Central Italy, i.e. Umbria (n=150), Marche (n=52) and Latium (n=98) (Fig. 1), in which heavy tick infestations associated with flu-like syndromes attributable to TBDs were frequently reported by veterinary practitioners and animal/farm owners. The study group consisted entirely of animals born and reared in Italy that had not traveled outside the country.

Blood samples were collected from the jugular vein in sterile tubes both with and without ethylenediaminetetraacetic acid (EDTA) and maintained at +4°C. The samples without EDTA were centrifuged at 4,000 rpm for 10 min, and the separated sera were stored at -20°C pending serological testing. The samples containing EDTA, on the other hand, were destined for DNA extraction.

Testing for serum IgG antibodies against *B. caballi*, *T. equi*, *B. burgdorferi* and *A. phagocytophilum*, was performed using the Immunofluorescence antibody test (IFAT). The test was run using commercial antigens (MegaScreen® FLUOBABESIA caballi/MegaScreen® FLUOTHEILERIA equi/MegaScreen® FLUOANAPLASMA ph/MegaScreen® FLUOBORRELIA horses) obtained from DIAGNOSTIK MEGACORE Laboratories (Horbranz, Austria) and a fluorescein-conjugated anti-horse antibody (rabbit-anti-horse-IgG-FITC, Sigma Immunochemicals, St. Louis, MO, U.S.A.), diluted 1:200 in Blue Evans solution. All samples were screened at an initial dilution of 1:80 in a phosphate-buffered saline solution (pH 7.2), as described in the manufacturer's protocol. Seropositive samples were subsequently diluted in order to determine the end-point titer. Positive and negative controls were included in each run. In the interest of excluding the possibility of a cross-reaction between *Borrelia* spp. and *Leptospira* spp., serum samples were also



Fig. 1. Regions where sampling has been carried out and number of samples for each Region.

tested using the SNAP® 4Dx test kit (IDEXX Laboratories, Westbrook, ME, U.S.A.), a qualitative in-clinic rapid ELISA test that specifically detects antibodies against the invariable domain IR6 of the variable surface antigen VlsE of *B. burgdorferi*, commonly known as the “C6 antigen”.

DNA was extracted from the blood samples containing EDTA using the QIAamp DNA Mini Kit (QIAGEN S.p.A., Milan, Italy) in accordance with the manufacturer's guidelines and then subjected to PCR assays previously described in veterinary literature: *A. phagocytophilum* DNA was detected using a nested-PCR protocol that amplifies a specific 928 bp fragment of the 16S-rRNA gene as described by Barlough *et al.* [2]; a specific nested PCR protocol [12] amplifying a 226–266 bp fragment (depending on the strains) encompassing the 5S–23S intergenic spacer region of the rRNA was used for *B. burgdorferi* DNA detection; in order to identify the presence of babesial parasite DNA, PCR was performed using the CRYPTO F [22] and RLB-R2 [8] that amplifies a fragment of the 18S-rRNA approximately 800 bp in size.

PCR assays were run with 50 µl of PCR reaction mixture containing 10 µl of 10× Tfl buffer (Promega, Milan, Italy), 1 µl of 10 mM dNTPs, 1 µl (10 pmol) of each primer, 3 µl of 25 mM MgSO<sub>4</sub>, 1 µl of DNA sample (80 ng/µl), 1 µl of Tfl Polymerase (Promega) and 32 µl of nuclease-free water per reaction. Amplification reactions were carried out in a ONE-Personal PCR Thermocycler (EuroClone, Milan, Italy).

In order to confirm amplicon identity, all obtained amplified fragments were purified from excess primers and buffers using the ExoSAP-IT (Affymetrix, Santa Clara, CA, U.S.A.) kit and prepared for sequencing with the ABI Prism BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystem,

Table 1. Prevalence (%) and 95% CI (lower and upper confidence intervals) of the equine tick-borne infections investigated using serological and molecular testing

Pathogens	IFAT	PCR
	No. of positive samples (Prevalence, CI)	No. of positive samples (Prevalence, CI)
<i>Theileria equi</i>	123 (41.0%, 15.0–85.3%)	35 (11.7%, 15.0–36.4%)
<i>Babesia caballi</i>	78 (26.0%, 13.2–65.1%)	20 (6.0%, 0.7–20.0%)
<i>Anaplasma phagocytophilum</i>	41 (13.4%, 1.7–40.5%)	20 (6.7%, 0.8–21.4%)
<i>Borrelia burgdorferi</i>	21 (7.0%, 0.8–22.1%)	7 (2.3%, 0.3–9.8%)

Table 2. Number of horses, prevalence (%) and 95% CI (lower and upper confidence intervals) of single and multiple equine tick-borne infections investigated using serologic and molecular testing

Pathogens		No. IFAT positive (prevalence, CI)	No. PCR positive (prevalence, CI)
Single infection	TE	60 (20.0%; 2.5–50.7%)	15 (5.0%; 0.6–16.9%)
	BC	19 (6.3%; 0.7–18.3%)	0
	AP	9 (3.0%; 0.4–12.4%)	12 (4.0%; 0.4–13.5%)
	BB	6 (2.0%; 0.3–8.7%)	1 (0.3%; 0.01–0.9%)
Double infection	TE+BC	44 (14.7%; 1.5–43.2%)	18 (6.0%; 0.7–18.3%)
	AP+BB	13 (4.3%; 1.6–16.9%)	6 (2%; 0.2–8.9%)
	TE+AP	2 (0.6%; 0.1–8.2%)	0
Triple infection	TE+BC+AP	15 (5.0%; 0.6–16.9%)	2 (0.6%; 0.1–8.2%)
	TE+AP+BB	2 (0.6%; 0.1–8.2%)	0

TE: *Theileria equi*; BC: *Babesia caballi*; AP: *Anaplasma phagocytophilum*; BB: *Borrelia burgdorferi*.

Foster City, CA, U.S.A.). In particular, the products were purified using the PERFORMA DTR Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD, U.S.A.) and sequenced with a 16-capillary ABI PRISM 3130 Genetic Analyzer (Applied Biosystem). SeqScape (Applied Biosystem) software was used in order to analyze the sequences; the forward and reverse sequences were aligned with ClustalW, validated visually, and consensus sequences were generated using the Applied Biosystems SeqScape Software 1 (version 2.5). The resultant consensus sequences were compared with sequence data in GenBank using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

**Statistical analysis:** Prevalence and 95% binomial confidence intervals (CI) were calculated for the serologic and molecular test results regarding each TBP.

Both concordant and discordant IFAT and PCR results were assessed by computing K statistics. K values <0.4, between 0.5 and 0.75 and >0.75 were considered as poor agreement, good agreement and excellent agreement, respectively [38]. All statistical analyses were performed using the WINPEPI (PEPI-for-Windows) computer programs for epidemiologists (Epidemiol. Perspect. Innov. 1:6. Available from: [www.epi-perspectives.com/content/1/1/6](http://www.epi-perspectives.com/content/1/1/6)).

## RESULTS

Despite frequent reports of tick infestation in the farms housing the animals during the months preceding sample collection, none of the horses participating in the study presented clinical signs of TBDs when the blood samples were taken.

The results of the serological and molecular tests performed on the horse blood samples are reported in Table 1.

Serologic testing using the IFAT test revealed a total of 78 (26.0%) and 123 (41.0%) horses out of the 300 analyzed to be seropositive for *B. caballi* and *T. equi*, respectively; meanwhile, 41 (13.4%) and 21 (7.0%) horses were, respectively, seropositive for *A. phagocytophilum* and *B. burgdorferi*. Serum end-point titers varied between 1:80 to  $\geq 1:1,280$  for *B. caballi*, *T. equi* and *A. phagocytophilum* and between 1:80 to 1:320 for *B. burgdorferi*. The entire group consisting of 21 *B. burgdorferi*-IFAT-positive horses also tested positive for antibodies using the SNAP® 4Dx test. Multiple seropositivities were observed in 76 horses (25.3%). Within this group, 59 (19.7%) horses tested positive for 2 and 17 (5.7%) for 3 species of equine TBPs, as shown in Table 2. The most common pathogen associations were between *T. equi* and *B. caballi*, occurring in 44 animals (14.7%), followed by coinfection with *T. equi*, *B. caballi* and *A. phagocytophilum* in 15 horses (5%) and coinfection with *A. phagocytophilum* and *B. burgdorferi* in 13 horses (4.3%). The PCR assays performed resulted in 54 (18.0%) horses testing positive for one or more TBD pathogen out of the 300 that were tested. In particular, 35 (11.7%) animals were PCR positive for *B. caballi*, 20 (6.0%) for *T. equi*, 20 (6.7%) for *A. phagocytophilum* and 7 (2.3%) for *B. burgdorferi* (Table 1). All sequenced PCR amplicons were confirmed by comparison with sequences published in GenBank and matched with 99–100% homology. Of the total of 54 PCR positive samples obtained, 28 (9.3%) resulted positive for single pathogens: 15 horses tested positive for *T. equi*, 12 for *A. phagocytophilum* and 1 for *B. burgdorferi*. Meanwhile,

Table 3. Summary and elaboration of IFAT and PCR results

Pathogen	Test	No. of positives	No. positive discordance *disagreement	No. negative discordance *disagreement	Positive agreement (%)	Negative agreement (%)	K value
<i>Theileria equi</i>	IFAT	123	96	8	34.2%	76.5%	0.32
	PCR	35					
<i>Babesia caballi</i>	IFAT	78	60	2	36.7%	87.6%	0.34
	PCR	20					
<i>Anaplasma phagocytophilum</i>	IFAT	41	21	0	65.6%	96.1%	0.62
	PCR	20					
<i>Borrelia burgdorferi</i>	IFAT	21	16	2	35.7%	96.9%	0.48
	PCR	7					

26 horses (8.7%) were found to be PCR positive for 2 or more tick-borne pathogens. In particular, 2 (0.6%) animals tested positive for 3 different TBPs (*T. equi*, *B. caballi* and *A. phagocytophilum*), while 24 (8%) were positive for 2 different TBPs; among these latter samples, 18 horses (6.0%) resulted to be simultaneously PCR positive for both *T. equi* and *B. caballi*, whereas 6 (2.0%) were PCR positive for both *B. burgdorferi* and *A. phagocytophilum*.

The concordance between IFAT and PCR results was expressed using K statistics. K values were 0.32 for *T. equi*, 0.34 for *B. caballi*, 0.62 for *B. burgdorferi* and 0.48 for *A. phagocytophilum* (Table 3).

## DISCUSSION

The current study represents an attempt to generate a systematic overview of the most important equine TBPs occurring in Central Italy. To date, serologic testing represents the most commonly used analytical method in epidemiological surveys, particularly those involving a large number of animals, even though it does not always provide sufficient information for diagnosis as an indirect means of testing for disease. In this study, it was therefore decided to incorporate PCR testing both as a comparative measure and in order to directly evidence the presence of TBPs within the surveyed population.

The overall seroprevalence (52.3%) and PCR positivity (18.0%) observed within the sampled population indicate a high level of exposure to TBPs of potential clinical importance in both veterinary and human medicine. The highest seroprevalence values were recorded for EP (*T. equi* 41.0%, *B. caballi* 26.0%). These findings are consistent with the prevalence rates previously described in Italian horses [4, 26, 27, 31, 39] and could be attributed both to the widespread diffusion of specific vectors (*Dermacentor*, *Hyalomma* and *Rhipicephalus*) [34], and to the role played by the horse as a reservoir for infection. The seroprevalence rates obtained in this study also resemble those reported in a cross-sectional study conducted on horses from Galicia, Spain, describing a prevalence of 40.0% for *T. equi* and 28.3% for *B. caballi*. (Chamaco *et al.*, 2005); conversely, lower prevalence rates of EP were reported in Swiss horses (Sigg *et al.*, 2010) [33] with a total of 4.8% for both *T. equi* and *B. caballi*.

The seroprevalence of *A. phagocytophilum*, obtained

in this study (13.4%), confirms the presence of this TBP in Central and Southern Italy [16, 17, 28] and suggests its possible association, in terms of endemic patterns, with *B. caballi* and *T. equi* [27, 32]. This value also coincides with data previously reported in other European countries, including France, Spain and Sweden [1, 17, 25].

Meanwhile, the seroprevalence rates for *B. burgdorferi* reported here (7.0%) were lower in comparison with values previously described in Germany (16.1%) [24], Sweden (16.8%) [17], Slovakia (47.8%) [36], Poland (25.6%) [35] and Denmark (29.0%) [21].

The differences between seroprevalence values described in this paper, should, however, be interpreted with caution considering that various factors may have played a role in their determination, including the horse populations surveyed, the geographical differences in vector exposure and the potential differences in the diagnostic methods employed.

In this study, prevalence rates determined using serological tests resulted higher in comparison with the results obtained using PCR assays; similar differences, expressed by moderate K values, have been observed in other studies and underline the significant discrepancy between the two testing methods [14]. These inconsistencies, stemming from the existence of seropositive yet PCR-negative animals, could be the expression of previous exposures to TBPs, subsequent immune responses and clearing of pathogens or may simply reflect the absence of detectable levels of bacteremia or parasitaemia.

On the other hand, PCR positive results in seronegative animals could indicate that the samples were obtained very early in the course of infection, prior to any detectable antibody response. Alternatively, these cases may reflect a failure to respond to acute TBP infection or more simply, a false negative result. Therefore, repeated serologic testing should be performed in order to monitor for seroconversion.

In the specific case of *B. burgdorferi*, PCR negative results do not rule out infection, as false-negative results can occur when the procedure is performed using DNA obtained from blood samples [6]. In practice, PCR testing of blood is usually performed in order to confirm infection in clinically suspect *B. burgdorferi* s.l. seropositive horses [6, 23] or during experimental studies [10]. Nevertheless, limited information is available regarding the diagnostic value of these tests in asymptomatic horses.

In conclusion, it has become increasingly apparent that TBP coinfections in the horse are common in several regions of the world [21, 42]. This study represents the first documentation of multiple equine TBP infections in Italy using both serologic and molecular methods. Specifically, multiple TBP exposures were ascertained using IFAT and PCR testing in 25.3 and 8.7% of the total sample population, respectively. The most common associations observed consisted of *T. equi* and *B. caballi* followed by *B. burgdorferi* and *A. phagocytophilum*.

The consequences of TBP co-infections in horses have not yet been well established. Simultaneous infection with multiple agents may account for some of the differences observed clinically in comparison with infections involving a single pathogen. A further research is, however, necessary in order to fully understand the characteristics of co-infection with multiple tick-borne organisms and assess the differences between single and multiple TBP infections.

In the present paper, an unprecedented degree of concurrent exposure to multiple TBPs was documented in Central Italian horses using serologic and molecular methods. Public and private health providers both in Italy and abroad, particularly veterinary practitioners and physicians, should be aware of the potential for tick-borne diseases in horses, especially where large numbers of animals are maintained for recreational purposes and many ticks are present.

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