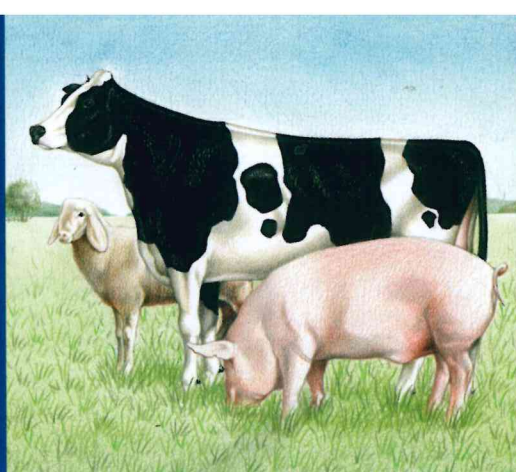


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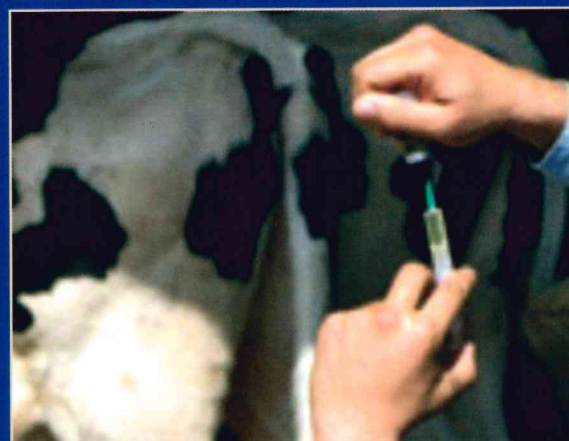
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- Molecular detection of *Theileria equi* in donkeys (*Equus asinus*) in a selected site in central Italy



Molecular detection of *Theileria equi* in donkeys (*Equus asinus*) in a selected site in central Italy



R. PAPINI¹, F. SALARI¹, G. ROCCHIGIANI¹, A. LEONI¹, G. RAGONA^{2a}, C. RONCORONI^{2b}, A. FAGIOLO^{2c}, V. VENEZIANO³, F. MANCIANTI¹

¹ Dipartimento di Scienze Veterinarie - Università di Pisa

² Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, Sedi di ^a Firenze, ^b Roma, ^c Latina

³ Dipartimento di Medicina veterinaria e Produzioni animali - Università degli Studi di Napoli Federico II

SUMMARY

Equine piroplasmiasis is among the most relevant tick-borne diseases of domestic and wild equids. Donkeys (*Equus asinus*) represent a potential reservoir for haemoparasites by harbouring tick-transmitted haemoparasites that can infect horses. We investigated the occurrence of *Babesia caballi* and *Theileria equi* in a donkey farm in the province of Grosseto (central Italy) to determine their prevalence of infection. For this purpose, conventional polymerase chain reaction (PCR) assays were carried out on blood samples from 109 donkeys. These included 85 females and 24 males as well as 36 young, 49 adult and 24 old animals. *B. caballi* and *T. equi* were detected by using primers that amplify an approximately 560 bp portion of the small-subunit ribosomal DNA of most *Babesia* and *Theileria* species. All PCR-positive samples were sequenced to determine the species of amplified *Babesia* and *Theileria* DNA. Sequencing data analysis revealed that 36 (33%, 95% CI: 24.2-40.9%) donkeys were positive for *T. equi* DNA. No samples were positive for *B. caballi* DNA. *T. equi* PCR-positivity drastically increased with age (from 0% to 46.9% and 54.2%) and was not significantly associated with the gender. These results highlight the high molecular prevalence of *T. equi* in a donkey farm of central Italy and support the role of donkeys as carriers and reservoirs of theileriosis for horses. The lack of *B. caballi* DNA needs further investigation.

KEY WORDS

Theileria equi, *Babesia caballi*, Donkeys, *Equus asinus*, central Italy.

INTRODUCTION

Equine piroplasmiasis (EP) is a tick-borne protozoal intraerythrocytic infection caused by *Theileria equi* (formerly *Babesia equi*) and *Babesia caballi*, belonging to the phylum Apicomplexa^{1,2}. These haemoparasites are endemic in many subtropical, tropical and temperate regions of Africa, Asia, central and southern America, and southern Europe^{1,2}. EP is included in the OIE list, affects all equid species (horses, mules, donkeys, zebras), and can occur in hyperacute, acute or chronic forms². Symptomatic animals may show anorexia, weakness, weight loss, poor exercise tolerance, fever, anaemia, icterus, haemoglobinuria, and in some cases even sudden death². Mares may also transmit *T. equi* by intrauterine infection throughout their breeding life leading to abortion, stillbirth, and serious neonatal EP with high economic losses to horse breeders in EP endemic areas¹.

EP is a major constraint to the international movement of equids because the introduction of carrier animals into areas where vector ticks, such as *Hyalomma* spp., *Rhipicephalus* spp., and *Dermacentor* spp. are present, can lead to an epizootic spread of the disease^{1,2}. In particular, donkeys may

usually become asymptomatic carriers and potential reservoirs of EP³. Some information on the prevalence of EP in donkeys are available in Italy^{4,5,6,7} but worrying seroprevalence values as high as 70.4% for *B. caballi*⁴ and 44.3% for *T. equi*⁵ have been reported in last years. Based on these reports, therefore, planning and implementation of surveillance programmes on agents of EP are urgently needed in donkeys in this country. The distribution of *T. equi* and *B. caballi* in Italian donkey farms has not been studied extensively. Here we report the molecular detection of *T. equi* in a herd of donkeys in a selected site in Tuscany (central Italy).

MATERIALS AND METHODS

Study area and animal population sampled

Between August and September 2015, a herd of 109 Amiata donkeys (*Equus asinus*) was examined in toto for *B. caballi* and *T. equi*. The herd was reared for milk production and onotherapy in a donkey farm located in the municipality of Scarlino (42°54'29"N 10°51'03"E) within the province of Grosseto (Tuscany, central Italy). The farm site was very close to the Tyrrhenian sea, with typical Mediterranean climate conditions, and surrounded by pinewoods. The composition of the donkey herd examined is shown in Table 1. The donkeys were managed in paddocks with open housing

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Roberto Amerigo Papini (roberto.amerigo.papini@unipi.it).

Table 1 - Composition of a donkey herd examined by polymerase chain reaction for *Babesia caballi* and *Theileria equi* DNA in a selected site in Tuscany (central Italy), according to sex and three age groups: young (1-2 years), adult (3-10 years), and old (11-24 years).

Age group	Sex		Total
	Females	Males	
1-2 years	20	16	36
3-10 years	45	4	49
11-24 years	20	4	24
Total	85	24	109

system that does not protect them from extreme weather conditions, and fed with poliphyta hay ad libitum. All the animals were apparently healthy and in good body conditions as determined by general clinical examination, and no ticks were seen on them at the time of sampling. They were periodically treated with fenbendazole (Panacur Equine Paste®) for deworming but there was no history of topical, oral or injectable treatment with acaricide/insecticides or endectoparasiticides.

Collection of samples and molecular procedures

Peripheral whole blood samples were collected from the jugular vein by syringe, transferred into EDTA tubes, labeled with animal identification number, kept refrigerated in a cooler bag, and transported to the laboratory on the day of collection. Upon arrival to the laboratory, labelled whole blood samples were conserved for maximum three days at +4°C. DNA was extracted from 200 µL of the blood samples using a commercial kit (Blood/Cultured Cell Genomic DNA Extraction Mini Kit™, Fisher Molecular Biology, Trevose, PA, USA) according to the manufacturer's instructions and kept at +4°C until use for polymerase chain reaction (PCR) assay. *B. caballi* and *T. equi* were detected using primers that amplify an approximately 560 bp portion of the small-subunit ribosomal DNA of most *Babesia* and *Theileria* species⁸. All PCR-positive samples were sequenced to determine the species of amplified *Babesia* and *Theileria* DNA.

Statistical analysis

Prevalence values were determined as number of positive animals for at least one of the two haemoparasites/number

Table 2 - Prevalence values (%) and 95% confidence intervals (95% CI) of *Theileria equi* DNA in donkeys in a selected site in Tuscany (central Italy) according to sex and age groups, as determined by polymerase chain reaction.

Variables	No. examined	No. positive	%	95% CI
Age group	1-2 years	36	0	0.0-0.0
	3-10 years	49	23	46.9-60.9
	11-24 years	24	13	54.2-74.1
Sex	Females	85	33 ^a	28.5-49.2
	Males	24	3 ^a	0-25.7
Total	109	36	33	24.2-41.9

^a The gender does not influence the positivity for *T. equi* infection but it is confused by the age, that is the real reason of the increased positivity in females (Mantel-Haenszel Odds Ratio, correcting the Odds Ratio for the confounder = 2.0690).

of examined animals × 100. Confidence intervals of 95% (95% CI) were calculated. Differences were compared according to sex and age groups by chi-square test (Table 2). *P* values <0.05 were considered significant. Odds ratio with the corresponding 95% CI was also calculated as a measure of the risk. To control for potential confounding factors, the Mantel-Haenszel formula was applied.

RESULTS

Thirty six blood samples were found to be PCR-positive. Sequencing revealed that no samples were positive for *B. caballi* DNA. Positivity rates for *T. equi* DNA and results of statistical analysis are shown in Table 2.

DISCUSSION

The clinical form of theileriosis can be diagnosed by peripheral blood smear examination, but in carrier donkeys it is very difficult to demonstrate the parasite in stained blood smears as the parasitaemia is extremely low. For diagnosis of such low grade infection in carrier animals, serological tests and DNA-based molecular diagnostic techniques are mandatory³. The molecular detection of *T. equi* in blood samples requires amplification of DNA from parasites that are physically present in the blood stream. *T. equi* was widespread in the donkey farm surveyed since 33% of the animals were found to be positive by PCR. The remarkable age related increase in positivity (from 0% to 46.9% and 54.2%) can be explained by the transmission dynamics of the protozoan. It has been previously reported that the risk of exposure to infestations with ticks infected by *T. equi* and to their bites has the tendency to increase with age in donkeys⁹. An initial statistical effect of the gender (chi-square=5.86, *P*=0.0155, Odds Ratio=4.44 [1.23-16.07]) was unexpected due to the similarity of management of females and males in the farm. However, the Mantel-Haenszel test revealed that the age was a confounding factor associated both with the increased risk of infection and with the sex of animals, as there was a considerably higher number of adult and old females than males of the same age groups (n=65 vs. 8) in the population composition.

It should be highlighted that *T. equi* commonly occurs in subclinical form in donkeys since clinical signs were not observed in any of the PCR-positive animals. The lack of clinical signs may represent the low pathogenicity of the *T. equi* strain involved in this report. However, it is noteworthy that immune suppression caused by management and/or corticoids treatment may induce increase of parasitemia and appearance of clinical manifestations in asymptomatic hosts².

Results of other surveys on EP in donkeys are summarized in Table 3. The present prevalence is the highest molecular detection rate

Table 3 - Summary of results of surveys carried out on *Theileria equi* and *Babesia caballi* infections in donkeys by molecular and serological tests, including reference number (Ref. no.), country, number of animals examined (No. examined), molecular prevalence (DNA %), seroprevalence, and acronyms of diagnostic tests in brackets.

Ref. no.	Country	No. examined	<i>Theileria equi</i>		<i>Babesia caballi</i>	
			DNA % (test)	Seroprevalence % (test)	DNA % (test)	Seroprevalence % (test)
4	Italy	43 (PCR ^a) 81 (IFAT ^b)	2.3 (PCR)	37 (IFAT)	0 (PCR)	70.4 (IFAT)
5	Italy	203	ND ^c	44.3 (IFAT)	ND	35.5 (IFAT)
6	Italy	122	16.3 (RT-PCR ^d)	39.3 (IFAT)	0.8 (RT-PCR)	47.5 (IFAT)
7	Italy	138	17.4 (RT-PCR)	40.6 (IFAT)	3.6 (RT-PCR)	40.6 (IFAT)
10	Brazil	88	31.8 (n-PCR ^e)	73.9 (ELISA ^f)	20.4 (n-PCR)	93.2 (IFAT)
11	Kenya	71	72 (n-PCR)	ND	0 (n-PCR)	ND
12	Iran	106	50.9 (M-PCR ^g)	ND	0 (M-PCR)	ND
13	Turkey	75	ND	4 (c-ELISA ^h)	ND	1.3 (c-ELISA)
14	Ethiopia	395	ND	55.7 (IFAT)	ND	13.2 (IFAT)
15	Kenya	314	ND	81.2 (c-ELISA)	ND	0 (c-ELISA)

^a Polymerase chain reaction; ^b immunofluorescence antibody test; ^c not determined; ^d real-time PCR; ^e nested-PCR; ^f enzyme linked immunosorbent assay; ^g multiplex-PCR; ^h competitive ELISA.

of *T. equi* infection in donkeys in Italy so far^{4,6,7}. It is also close to the prevalence value reported in Brazil¹⁰ and lower than values observed in Kenya¹¹ and Iran¹². The present lack of *B. caballi* DNA is in agreement with results of studies carried out in some Italian regions^{4,6}, Kenya¹¹, and Iran¹² where molecular prevalences of 0-0.8% were found in donkeys. Nonetheless, molecular detection of *B. caballi* was also reported in 20.4%¹⁰ and 3.6%⁷ of donkeys in Brazil and Italy again, respectively.

Most of those studies combined molecular and serological methods^{4,6,7,10}. Seroprevalence values of *T. equi* and *B. caballi* varied from 37% to 44.3% and 35.5% to 70.4% in Italy^{4,5,6,7} or from 4% to 81.2% and 0% to 93.2% in other countries^{10,13,14,15}, respectively. Variations between studies performed in different countries or within the same country may be due to differences in number and population composition of animals sampled, environmental conditions, geographical areas, sampling techniques, study designs, diagnostic methods and so forth but mostly to differences in the management of donkeys, since adoption of strategic preventive measures against vector tick infestation is a pivotal factor in the control of all the tick-borne agents, including *T. equi* and *B. caballi*¹.

CONCLUSION

Our results show that, in areas with endemic stability, a high proportion of donkeys may be infected by *T. equi* with no cli-

nical evidence. This underlines the utility of introducing a surveillance system for the infection in any donkey herd, considering that different prevalences exist because risk factors are irregularly distributed.

The high molecular prevalence of *T. equi* infection in the herd of donkeys examined in this survey is most likely to be linked to the total lack of any rigorous tick control program in the farm. Therefore, the animals were exposed to ticks infected by *T. equi* during their lifetime and *T. equi* transmission occurred in the herd. Currently, there is no suitable pharmacotherapy available to clear the infection from affected donkeys³. Given that theileriosis may remain as a lifelong infection¹, it is likely that the positive donkeys of our study can act as carriers and reservoirs of *T. equi* for all susceptible hosts throughout their life³. Carrier animals remain a source of infection not only transmitting the parasite to vector ticks but also through veterinary procedures such as blood transfusions, dental treatments and stomach tubing¹.

On the other hand, the absence of *B. caballi* infection might be due to a limited distribution of this haemoprotozoan in the study area or, alternatively, it is possible that *B. caballi* may persist in subclinical form just for a period of time in donkeys and subsequently be naturally eliminated, as observed in horses¹. Although *B. caballi* infections have lower frequency than *T. equi* infections in most regions¹, the lack of *B. caballi* DNA in donkeys of our study needs further large scale epidemiological studies to allow definitive conclusions.

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