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Short Communication

Seroprevalence of *Toxoplasma gondii* and *Leishmania* spp. in domestic donkeys from Portugal

Soroprevalência de *Toxoplasma gondii* e *Leishmania* spp. em jumentos domésticos de Portugal

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Abstract

Toxoplasma gondii and *Leishmania infantum* are zoonotic protozoal parasites. Serum samples were obtained from 186 donkeys (*Equus africanus asinus*) from Portugal and assessed for antibodies to *T. gondii* by the modified agglutination test (MAT). For titration of antibodies to *Leishmania* spp. the direct agglutination test was used (DAT). Eleven donkeys were seropositive for *T. gondii* with titres of 20 (n = 7), 80 (n = 2), 640 (n = 1) and ≥ 2560 (n = 1). One donkey was seropositive for *Leishmania* spp. (titre of 800). Donkeys in Portugal are exposed to and can be infected with *T. gondii* and *Leishmania* spp.

Keywords: Direct agglutination test, donkey, *Leishmania infantum*, modified agglutination test, *Toxoplasma gondii*, Portugal.

Resumo

Toxoplasma gondii e *Leishmania infantum* são protozoários parasitas com potencial zoonótico. Foram obtidas amostras de soro de 186 jumentos (*Equus africanus asinus*) e avaliadas para anticorpos anti-*T. gondii* pelo teste de aglutinação direta modificada (TADM), em Portugal. Para a titulação de anticorpos anti-*Leishmania* spp. foi usado o teste de aglutinação direta (TAD). Onze jumentos foram soropositivos para *T. gondii* com títulos de 20 (n = 7), 80 (n = 2), 640 (n = 1) e ≥ 2560 (n = 1). Um jumento foi soropositivo para *Leishmania* spp. (título de 800). Os jumentos em Portugal estão expostos e podem ser infectados com *T. gondii* e *Leishmania* spp.

Palavras-chave: Teste de aglutinação direta, jumento, *Leishmania infantum*, teste de aglutinação direta modificada, *Toxoplasma gondii*, Portugal.

Toxoplasma gondii and *Leishmania infantum* are zoonotic protozoa of global veterinary and medical importance, which can infect a variety of vertebrate hosts, including donkeys (*Equus africanus asinus*). Wild and domestic felids are the definitive hosts of *T. gondii*. The ingestion of food or water contaminated with sporulated oocysts or that of tissue cysts in undercooked meat are two major routes of postnatal transmission of *T. gondii*. Up to one third of the world human population has been estimated to be exposed to this parasite (DUBEY, 2010).

Zoonotic leishmaniosis caused by *L. infantum* is endemic in the Mediterranean basin, including Portugal, with domestic dogs as the primary reservoir and phlebotomine sand flies as vectors (CARDOSO et al., 2012). Infection in humans may result in a broad range of diseases from severe visceral to mild cutaneous clinical outcomes, whereas canine leishmaniosis is a frequent chronic viscerocutaneous and even fatal disease (WHO, 2010).

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Cases of cutaneous leishmaniosis in horses have been reported in southern (SOLANO-GALLEGO et al., 2003; GAMA et al., 2014) and Central Europe (MÜLLER et al., 2009).

The donkey is important and useful to humans in many ways. In some European countries, institutions and breeder associations have made strong efforts to preserve the existing autochthonous breeds, promoting their use as pets, in recreational activities (agritourism and trekking), for onotherapy (especially with children), meat products (gourmet and dietary products) and milk production (cosmetic industry and human nutrition) (MACHACOVA et al., 2014).

Between February 2015 and February 2016, blood samples were obtained from 186 apparently healthy donkeys living in seven out of 18 districts of mainland Portugal. Most (102; 54.8% of 186) were from northern districts (Bragança, n = 97; Porto, n = 5) and 84 (45.2%) donkeys were from southern districts (Santarém, n = 12; Lisboa, n = 6; Setúbal, n = 16; Évora, n = 1; and Faro, n = 49) of the country.

The animals were randomly sampled, by venipuncture of a jugular into tubes without anticoagulant, and data obtained via personal interviews with the owners, by using a questionnaire. Information included sex, age, breed, activity, type of housing and contact with other animal species (Table 1). The Chi-square or Fisher's exact tests were used to compare seropositivity values and a probability (p) value < 0.05 was considered as statistically significant.

This study complied with the Portuguese legislation for the protection of animals (Law no. 92/1995 and Decree-Law nº 113/2013). Owners provided their informed consent for inclusion of their animals in the study.

Serum samples were assessed for anti-*T. gondii* Ig G antibodies by a modified agglutination test (MAT) commercial kit (Toxo-Screen DA® bioMérieux, Lyon, France) as per the manufacturer's instructions. Samples were tested at dilutions 1:20, 1:40, 1:80, 1:640 and 1:2560. MAT positive results had visible agglutination (at least 50% of the well's diameter) after 5-18 hours of incubation at room temperature. A cut-off titre of 20 (2UI/ml in relation to a World Health Organization international reference serum) was used (LOPES et al., 2013).

Table 1. General data of the 186 donkeys studied and seroprevalence of *Toxoplasma gondii*.

Variable/categories	Nº of donkeys (%) tested	Percentage (n) of seropositive	CI
Sex		p = 1.0	
Female	118 (63.4)	5.9 (7)	2.4-11.8
Male	68 (36.6)	5.9 (4)	1.6-14.4
Age class		p = 0.501	
[2-11] months	13 (7.0)	0.0 (0)	0.0-24.7
[1-5[years	40 (21.5)	5.0 (2)	0.6-16.9
[5-14[years	81 (43.5)	8.6 (7)	3.5-17.0
[14-32] years	52 (28.0)	3.8 (2)	0.5-13.2
Breed		p = 0.210	
Asinina de Miranda	115 (61.8)	7.8 (9)	3.6-14.3
Mixed	71 (38.2)	2.8 (2)	0.3-9.8
Aptitude		p = 0.532	
Leisure	107 (57.5)	4.7 (5)	1.5-10.6
Work	79 (42.5)	7.6 (6)	2.8-15.8
Housing		p = 0.188	
Indoors and Mixed	63 (33.9)	9.5 (6)	3.6-19.6
Outdoors	123 (66.1)	4.1 (5)	1.3-9.2
Geographical area		p = 1.0	
North	102 (54.8)	5.9 (6)	2.2-12.4
South	84 (45.2)	6.0 (5)	2.0-13.3
Contact with cats		p = 0.082	
No	54 (29.0)	11.1 (6)	4.2-22.6
Yes	132 (71.0)	3.8 (5)	1.2-8.6
Contact with donkeys		p = 0.091	
No	9 (4.8)	22.2 (2)	2.8-60.0
Yes	177 (95.2)	5.1 (9)	2.4-9.4
Contact with dogs		p = 0.015	
No	29 (15.6)	17.2 (5)	5.8-35.8
Yes	157 (84.4)	3.8 (6)	1.4-8.1
Total	186 (100)	5.9 (11)	3.0-10.3

CI: 95% confidence interval.

The direct agglutination test (DAT) for titration of immunoglobulin g (IgG) antibodies specific to *Leishmania* spp. followed a predefined protocol (SCHALLIG et al., 2002), using a standard freeze-dried antigen at a concentration of 5×10^7 promastigotes per millilitre (Academic Medical Center, Amsterdam, The Netherlands). Two-fold dilutions series ranging from 1:25 to 1:1600 were tested and a cut-off titre of 200 was assumed for seropositivity (LOPES et al., 2013; GAMA et al., 2014).

The median age of donkeys was 8 years (interquartile range: 12-15 years). The results of specific serology for *T. gondii* are summarized in Table 1. The 11 donkeys seropositive for *T. gondii* had titres of 20 (n = 7), 80 (n = 2), 640 (n = 1) and ≥ 2560 (n = 1). The youngest donkey seropositive for *T. gondii* was 3 years old and the oldest one 17 years. Except for contact with dogs (Table 1), no other statistically significant differences were found relating to seropositivity for *T. gondii* among the categories of the same variable. One donkey (0.5%; CI: 0.0-3.0%) was found seropositive for *Leishmania* spp. with a titre of 800. This animal was a 32-year-old mixed-breed female from southern Portugal (Figure 1), used for work and housed exclusively outdoors that had been in contact with other animals, including cats, donkeys and dogs. The *Leishmania* spp.-seroposive donkey was seronegative for *T. gondii* (MAT titre < 20). A statistically significant difference (p = 0.003) was found between the seroprevalence values of *T. gondii* (5.9%) and *Leishmania* spp. (0.5%).

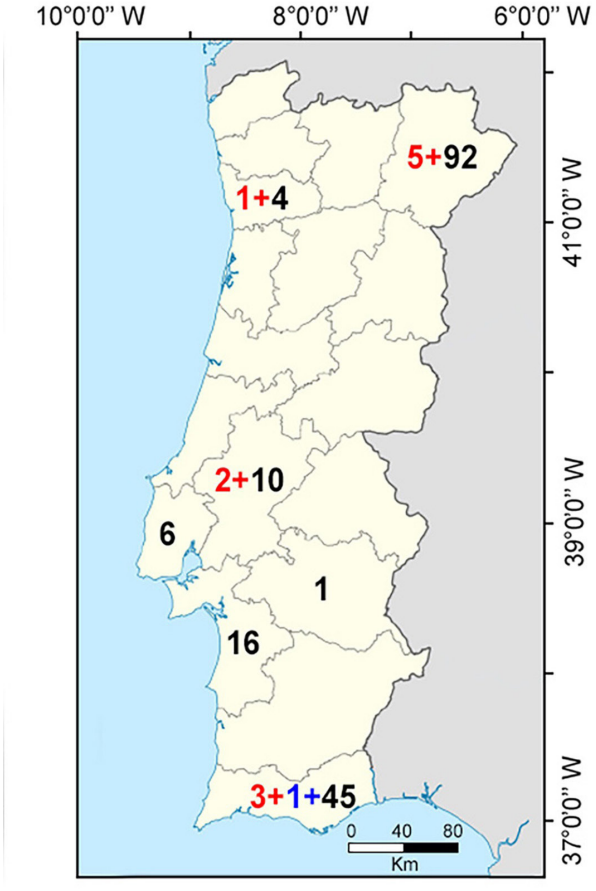

Figure 1. Map of mainland Portugal depicting the seven sampled districts with the numbers of donkeys seropositive for *Toxoplasma gondii* (red), seropositive for *Leishmania* spp. (blue) and seronegative for both (black).

Exposure and infection seem to be homogeneously distributed throughout the country. Previous studies in Portugal have shown a widespread distribution of antibodies to *T. gondii* in humans, and animal species (LOPES et al., 2014). However, this is the first epidemiological investigation of *T. gondii* in donkeys in Portugal. The seroprevalence of *T. gondii* in donkeys (5.9%) was lower (p = 0.017) than the 13.3% previously reported in horses from northern Portugal (LOPES et al., 2013). In other studies from this geographical region, seroprevalence values were found to be 35.8% (p < 0.001) in cats (LOPES et al., 2008), 38.0% (p <0.001) in dogs (LOPES et al., 2011) and 24.4% (p < 0.001) in women at their child-bearing years (LOPES et al., 2012).

There are several serologic surveys for *T. gondii* infection in donkeys worldwide, with prevalence ranging from 5% in Italy (MACHACOVA et al., 2014) to 65.6% in Egypt (EL-GHAYSH, 1998). Some of the differences in seroprevalences can be probably related to the number of donkeys tested and the different serological tests used, but also to feeding conditions, animal husbandry practices and the prevalence of *T. gondii* in cats (DUBEY et al., 2014; ALVARADO-ESQUIVEL et al., 2015). In the present study, seroprevalence of *T. gondii* (5.9%) was significantly lower (p < 0.001) than the 25.6% found also by means of the MAT in 82 donkeys from southern Spain (GARCÍA-BOCANEGRA et al., 2012).

In donkeys that had contact with dogs a significantly lower seropositivity (3.8%) for *T. gondii* was observed (Table 1). As per information according to the owners, dogs mentioned in this study were used for guard or for companion purposes. Under a binomial categorization (i.e. "no" versus "yes") the "contact with cats" variable was not found to be statistically associated with seropositivity to *T. gondii* in donkeys (Table 1). Nevertheless, the presence of dogs among donkeys might contribute to decrease the frequency of contacts between donkeys and cats, and to reduce exposure of the former to oocysts shed in the environment by the latter. Regarding sex, in the USA, by using the MAT, the seroprevalence values of *T. gondii* (7.0% versus 4.1%) were also found not to be significantly different between 282 female and 91 male donkeys, respectively (DUBEY et al., 2014). A similar situation was found in Italy by means of IFAT, i.e. seroprevalence was 8.7% in 207 females and 3.2% in 31 male donkeys (p = 0.559) (MACHACOVA et al., 2014).

In the present study, all *T. gondii* seropositive donkeys (n = 11) were 3 to 17 years old, a circumstance which suggests postnatal infection or exposure. This also provides evidence for an increased risk of infection with age, based on a longer contact with oocysts from the environment (BOUGHATTAS et al., 2011).

The results of the present study indicate that donkeys in Portugal are exposed to *T. gondii*. The voluntary consumption of equid meat by people is not a common habit in this country, but seroprevalence studies in domestic donkeys elsewhere suggest that the consumption of undercooked or raw meat from these animals

represent a potential source of infective *T. gondii* cysts for humans (ALVARADO-ESQUIVEL et al., 2015).

In some parts of the world, donkey milk is used for human nutrition. In fact, the use of jenny milk for people allergic to cow milk is becoming increasingly popular (DUBEY et al., 2014). A recent study from Italy suggests the transmission of *T. gondii* following consumption of raw milk from jennies (MANCIANTI et al., 2014).

Antibodies to *Leishmania* spp. were detected in only one (0.5%) out of the 186 donkeys in the present study. Zoonotic leishmaniosis caused by *L. infantum* is endemic in Portugal, with dogs acting as primary reservoirs (CARDOSO et al., 2012), but both infection and disease have also been observed in cats (PIMENTA et al., 2015) and horses (LOPES et al., 2013; GAMA et al., 2014). The prevalence of antibodies to *Leishmania* spp. in donkeys (0.5%) found in the present study is lower ($p = 0.031$) than the 4.0% homologous prevalence previously reported in horses from northern Portugal (LOPES et al., 2013). Seroprevalence was 3.6% ($p = 0.020$) in apparently healthy and 18.6% ($p < 0.001$) in clinically suspect dogs (CARDOSO et al., 2012), and 2.8% ($p = 0.037$) in cats (CARDOSO et al., 2010) also from northern Portugal. The female donkey found seropositive in the present study was born and lived in southern Portugal. In this geographical area, seroprevalence values were found to be 3.8% ($p = 0.023$) in apparently healthy and 27.7% ($p < 0.001$) in clinically suspect dogs (CARDOSO et al., 2012), and 3.7% ($p = 0.015$) in cats (MAIA et al., 2015b).

Phlebotomine sand fly insects are the vectors of *Leishmania* spp. in Europe, with one of the vector species for *L. infantum* being *Phlebotomus perniciosus*, which is present in southern Portugal (SCHREY et al., 1989) and can feed on blood from several mammalian species, including donkeys (BRAVO-BARRIGA et al., 2016). Rodents, horses, chicken, rabbits, pigs, cats, cattle and sheep were found to be a blood source for sand flies in southern Portugal (MAIA et al., 2013, 2015a).

The donkey found seropositive for *Leishmania* spp. was apparently healthy, a circumstance which is compatible with the report that experimental infection of donkeys in Brazil with *Leishmania chagasi* (syn. *L. infantum*) did not originate disease (CERQUEIRA et al., 2003). The role of infected donkeys as potential secondary reservoirs of *Leishmania* spp. should be further investigated worldwide in areas where leishmaniosis is endemic.

In conclusion, this is the first epidemiological survey on *T. gondii* and *Leishmania* spp. in donkeys from Portugal, where these animals are exposed to and may be infected with those zoonotic parasitic protozoa.

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