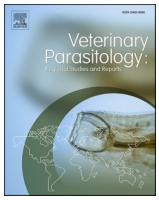
Veterinary Parasitology: Regional Studies and Reports 54 (2024) 101093

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr



Original Article

Serological and molecular detection of *Toxoplasma gondii* infection in apparently healthy horses in eastern of Spain

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ARTICLE INFO

Keywords:
Horses
Molecular detection
Seroprevalence
Spain
Toxoplasma gondii

ABSTRACT

Toxoplasmosis is one of the most common parasitic zoonoses and represents a significant health risk for humans, especially for immunodeficient patients. The main transmission route is by oral uptake of oocysts and consumption of undercooked meat of infected animals. Different species have been evaluated as possible reservoirs of the parasite, but few studies have been carried out to examine the role of horses in transmission of the disease. Given the proximity of these animals to humans and the widespread consumption of their meat in many countries, including the Mediterranean basin, it is important to determine the prevalence of *T. gondii* infection in this species. In this study, blood samples from 105 horses were collected and the presence of *T. gondii* was evaluated by serological and molecular methods. Antibodies against *T. gondii* of 12 horses (11.43%) were detected by enzyme-linked immunosorbent assay (ELISA), whereas 29 horses (27.62%) showed positive for PCR. Seroprevalence was related to use of the animals, being higher in horses used for dressage than in others. Purebreds had higher seroprevalence than crossbred animals. No differences between breed, sex or age were found. The results of this study confirm the presence of *T. gondii* infection in horses, highlighting the need to analyse the meat of this species before human consumption and to control of this infection in horses, as they could be an important reservoir of this zoonotic parasite.

1. Introduction

Toxoplasma gondii is an intracellular zoonotic parasite that causes the toxoplasmosis disease. In human, toxoplasmosis is the second cause of death among foodborne illnesses (Scallan et al., 2011). Symptoms can manifest long after primary infection, and their severity depends on the strain and the susceptibility of the host (Aguirre et al., 2019). Although cats and other felids are definitive hosts of *T. gondii*, several species of mammals can serve as intermediate hosts, including wild animals (Almeria et al., 2021; Dubey et al., 2021), livestock animals such as swine (De Berardinis et al., 2017), sheep (Dubey et al., 2008), goats (Dubey et al., 2011), chicken (Shokri et al., 2017), ducks, geese (Konell et al., 2019) and cattle (Belluco et al., 2016). In horses, different studies show high levels of *T. gondii* infection around the world (Dubey et al., 2020).

Horse meat consumption is still a very common custom in certain countries, with Spain the major producer in the EU (17%), followed by Italy (16%), Romania (14%), Poland (11%) and France (8.2%) (FAOSTAT, 2024). However, the data on prevalence infection of *T. gondii* in these countries are scarce. A recent study carried out by Cano-Terriza et al. (2023) in 1399 horses showed a seroprevalence of 18.9%, the most elevated data being found in Italy (27.1%) and Spain (16.6%). According to these data, the seroprevalence was found at around 16.3% in the Mediterranean area of Spain (Pala et al., 2024).

The aim of this study was to determine the prevalence of *T. gondii* infection by serological and molecular methods and identify risk factors associated with the exposure to this protozoan in horses in eastern Spain.

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https://doi.org/10.1016/j.vprsr.2024.101093
Received 10 May 2024; Received in revised form 14 July 2024; Accepted 1 August 2024
Available online 3 August 2024

2. Material and methods

2.1. Animals, epidemiological data and sample collection

A total of 105 horses (*Equus caballus*) living in rural equestrian centres of eastern Spain (Mediterranean basin) were included in the study. None of the animals presented any clinical signs compatible with *T. gondii* infection, and all animals were apparently healthy. For all horses, epidemiological data such as sex (male, female), reproductive status in males (castrated or not), age (foal, less than five years old; young, between five and twelve years old; adult, between thirteen and twenty-one years old; and elder, more than twenty-one years old), purebred or crossbred, housing system (outdoor, indoor), use (teaching, breeding, dressage, walking and jumping) and season of recovery samples (winter or spring). Breeds were classified based on their morphological types as mesomorphic, meso-brachymorphic, mesodolichomorphic or dolichomorphic (Dall'Olio et al., 2010), except ponies.

Samples were recovered from December 2022 to June 2023, in two periods (December 2022–January 2023 (winter), and May–June 2023 (spring)) in the Mediterranean region of Spain. For each animal, ten millilitres of whole blood were aseptically collected via jugular venipuncture using two Vacutainer tubes, one with anticoagulant EDTA and another without anticoagulant. The samples were kept at room temperature and the tubes without anticoagulant were centrifuged at 3000 rpm for 10 min to separate the serum, which was then transferred into cryovials, labelled and stored at −80 °C until use. The tubes with anticoagulant EDTA were used for DNA extraction for 24 h after extraction collection.

2.2. Serological analysis

Serum samples were used to determine the presence of specific IgG antibodies for *T. gondii*. Presence of antibodies was checked using the ELISA commercial kit ID Screen® Toxoplasmosis Indirect Multi-Species (TOXO-MS, Innovative Diagnostics, Grables, France), following the manufacturer's instructions. This test uses the P30 *T. gondii* tachyzoite surface protein as antigen. A sample-to-positive ratio (S/P%) was calculated for each serum sample according to $S/P\% = \frac{ODsample - OD\ NC}{OC\ PC - OD\ NC}$ x 100, where OD is the optical density either of the samples, the positive controls (PC) or the negative controls (NC). Animals with S/P% ≤ 40% were considered negative, inconclusive if 40% < S/P% < 50%, and positive with S/P%≥ 50%. Samples with results that were inconclusive were reanalysed. If the result was inconclusive a second time, they were withdrawn from the study. The overall sensitivity and specificity percentages of this ELISA test were 76.5% (CI 95%: 60.0–87.6%) and 87.7% (CI 95%: 76.7–93.9%), respectively (Bellatreche et al., 2022).

2.3. DNA extraction and molecular detection

Total gDNA was extracted for 200 μL of whole blood samples of EDTA tubes with PureLink™ Genomic DNA MiniKit (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions. The quality and quantity of DNA extracted was analysed by measure of absorbance ratios in NanoDrop One (ThermoFisher Scientific, Waltham, MA, USA). Only samples with A260/A280 ratio > 1.8 were used for the molecular detection.

A real-time PCR method targeting the 529-bp repeated fragment of *T. gondii* genome was used for molecular detection. PCR was performed in a 20 μL final volume containing 10 μL of master mix 2×, 10 pmol of each primer forward and reverse (AF1: CACAGAAGGGACAGAAGT and AF2: TCGCCTTCATCTACAGTC, respectively), 5 pmol of the labelled TaqMan probe (AF529: 6FAM-CTCTCCTCCAAGACGGCTGG- BHQ), and 40 ng of DNA template. The PCR protocol was performed as 2 min incubation at 50 °C, a first step denaturation at 95 °C for 10 min, and 40 cycles of a 2-step amplification (denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s) (Marino et al., 2017). Animal samples previously evaluated by a reference laboratory were used as positive control and molecular grade water was used as negative control.

2.4. Statistical analysis

Prevalence of parasitic infection was analysed using Pearson's Chi-square test to determine the association of seroprevalence of parasitic infection studied and putative risk factors. Seroprevalence and positive molecular detection for *T. gondii* were considered as dependent variables. Sex, reproductive status in males, age, purebred or crossbred, housing system, use, morphological types of equine breed and season (winter or spring) of the recovery sample were the independent variables analysed. Differences were considered statistically significant at p-value <0.05.

3. Results

Epidemiological data on horses included are shown in Table 1. Briefly, 41 females and 64 males (41 of them, castrated) were included. Of the 105 horses, 11 were foals, while 19 were young horses and 54 were adults, whereas 33 of them were crossbreeds. The most abundant morphology according to the equine breed was mesodolichomorphic (61.9%), followed by mesomorphic (1.9%), and meso-brachymorphic (1.9%). Three ponies were also included. Some 80% of the total animals had outdoor access and the most common use was dressage.

Seroprevalence of *T. gondii* infection in horses was found to be 11.43%, whereas positive molecular detection was found in 29 of horses included (27.62%). Only four horses were positive with both techniques (Table 2). Purebred horses presented higher seroprevalence than crossbreeds (Fig. 1, $p < 0.05$), but this difference was not observed in molecular detection. The same occurred in the "use" variable, with higher seroprevalence in animals used for dressage, followed by breeding and jumping (Fig. 2, $p < 0.05$). No effect was found in the remaining epidemiological variables analysed for prevalence of *T. gondii* infection, measured by ELISA (Table 3) or PCR technique (Table 4).

4. Discussion

The aim of this study was to determine the prevalence of *T. gondii*

Table 1
Epidemiological data of horses included in this study.

Variable			n	Percentage
Gender	Male ($n = 64$, 60.95%)	Castrated	41	39.05
		No castrated	23	21.90
	Female		41	39.05
Age	Foal		11	10.48
	Young		19	18.10
	Adult		54	51.43
	Elder		21	20.00
Purebred	Yes ($n = 72$)	Mesodolichomorphic	65	61.90
		Mesomorphic	2	1.90
		Mesobrachymorphic	2	1.90
		Ponies	3	2.86
	No		33	31.43
Access to outside	Yes		84	80.00
	No		21	20.00
Use	Teaching		26	24.76
	Breeding		4	3.81
	Dressage		66	62.86
	Walking		1	0.95
	Jumping		8	7.62
Season	Winter		45	42.86
	Spring		60	57.14

Table 2
Prevalence of *T. gondii* infection in horses using two different techniques.

		ELISA technique		Overall (%)
		Positive horses (%)	Negative horses (%)	
PCR technique	Positive horses (%)	4 (3.81)	25 (23.81)	29 (27.62)
	Negative horses (%)	8 (7.62)	68 (64.76)	76 (72.38)
Overall (%)		12 (11.43)	93 (88.57)	105 (100.00)

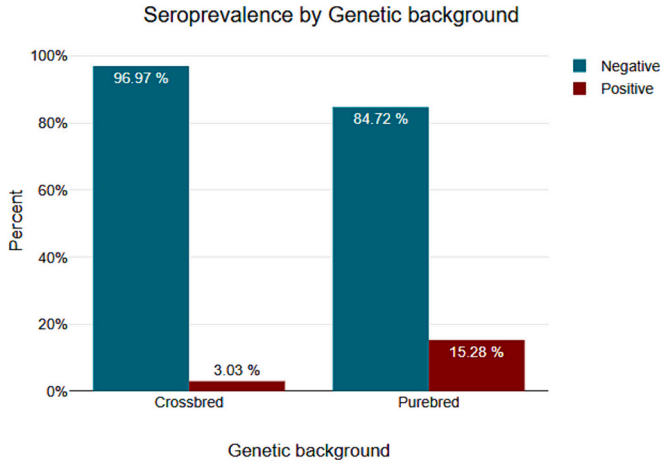

Fig. 1. Seroprevalence in purebred and crossbred horses.

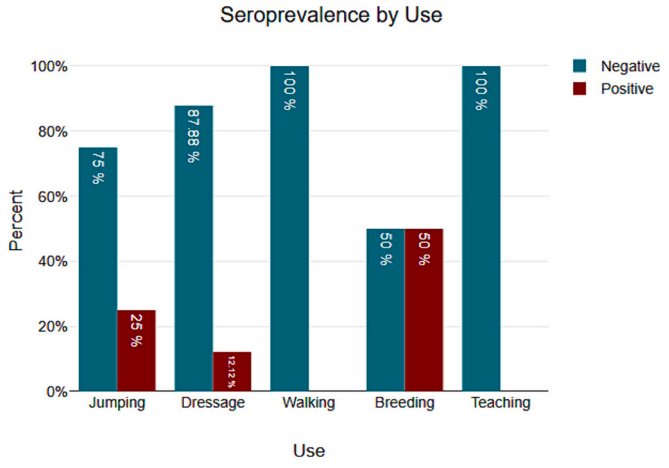

Fig. 2. Seroprevalence according to use of horses.

infection in horses by serological and molecular methods in eastern Spain and define the associated risk factors. The results showed seroprevalence of 11.43%, whereas molecular detection was found in 27.62% of animals included. Other authors have found similar results in seroprevalence of *T. gondii* infection in Mediterranean countries, such as 13.29% in Portugal (Lopes et al., 2013) or 17.20% in Italy (Papini et al., 2015). In Spain, similar results were found by García-Bocanegra et al. (2012) and Pala et al. (2024) in the southern and eastern regions of Spain. Studies on molecular detection in blood samples from horses were scarce. However, several studies have determined the infection in meat using molecular methods. In Tunisia, the prevalence of *T. gondii* in meat has been found at 39.7% (Amairia et al., 2023) and in France this data increases up to 43% (Aroussi et al., 2015). However, the low number of

Table 3
Seroprevalence and epidemiology of *T. gondii* infection according to related factors included.

Variable		Number of positives (%)	*p*-value
Gender	Male	8 (12.50)	0.6664
	Female	4 (9.76)	
Reproductive status in males	Castrated	4 (9.76)	0.3755
	No castrated	4 (17.39)	
Age	Foal	2 (18.18)	0.7581
	Young	3 (15.79)	
	Adult	5 (9.26)	
	Elder	2 (9.52)	
Genetic background	Purebred	11 (15.28)	< 0.05
	Crossbred	1 (3.03)	
Use	Teaching	0 (0.00)	< 0.05
	Breeding	2 (50.00)	
	Dressage	8 (12.12)	
	Walking	0 (0.00)	
	Jumping	2 (25.00)	
Access to outside	Yes	10 (11.90)	0.759
	No	2 (9.52)	
Season	Winter	5 (11.11)	0.9294
	Spring	7 (11.67)	

Table 4
Prevalence by PCR and epidemiology of *T. gondii* infection according to related factors included.

Variable		Number of positives (%)	*p*-value
Gender	Male	15 (23.43)	0.2312
	Female	14 (34.15)	
Reproductive status in males	Castrated	11 (26.89)	0.3924
	No castrated	4 (17.39)	
Age	Foal	3 (27.27)	0.4899
	Young	6 (31.58)	
	Adult	17 (31.48)	
	Elder	3 (14.29)	
Genetic background	Purebred	20 (27.78)	< 0.05
	Crossbred	9 (27.28)	
Use	Teaching	7 (26.92)	< 0.05
	Breeding	2 (50.00)	
	Dressage	16 (24.24)	
	Walking	0 (0.00)	
	Jumping	4 (50.00)	
Access to outside	Yes	25 (29.76)	0.326
	No	4 (19.05)	
Season	Winter	11 (24.44)	0.5286
	Spring	18 (30.00)	
Purebred	Yes	20 (27.78)	0.5537
	No	9 (27.28)	

animals included in this study is a limitation, so further studies analysing different seroprevalence between regions are necessary.Serological detection of *T. gondii* relies on the detection of IgM or IgG antibodies. The IgM antibodies indicate acute infection, but low levels of these antibodies can be found long after the acute phase of the disease (Del Bono et al., 1989). The peak of IgG antibodies is, typically, within one or two months after infection and declines at various rates (Holec-Gąsior and Sołowińska, 2022). Thus, determination of IgG antibodies to *T. gondii* is the most common serological method to detect active infection. Parasitic DNA remains in blood or meat sample for longer than IgG antibodies, and PCR is a more sensitive method (Marino et al., 2017). Therefore, the detection of IgG antibodies could indicate a recently active infection, although the IgG could persist lifelong at residual titters depending on the individual (Dupont et al., 2021; Voyiatzaki et al., 2024). The presence of IgG indicates immune response exclusively. However, the presence of parasite DNA indicates that the individual has been exposed to it at some point in its life, so if we only consider the results using serological methods, we could be

underestimating the prevalence of *T. gondii* (Abd El-Latif et al., 2023; Holec-Gąsior and Sołowińska, 2023). This data suggests that exposure to the parasite in horses is much greater than that reflected by studies carried out using serological techniques, which could explain the higher prevalence found in both blood and meat samples in different studies. To date, no correlation studies between molecular results observed in blood and meat samples have been carried out.

Regarding risk factors associated with seroprevalence of *T. gondii*, our results indicate that purebred animals have high prevalence of infection. Although the number of animals included in the study is low for some categories, the results obtained are in accordance with previous studies. Other authors have found the effect on equine breed in seroprevalence of *T. gondii* infection. For example, Marzok et al. (2023) found higher seroprevalence in thoroughbred horses than in crossbreeds, whereas Ouslimani et al. (2019) observed an effect of equine breed. On the contrary, the breed effect has not been observed in the determination of *T. gondii* infection by molecular methods. Overall Ig concentration is determined by equine breed and transcriptional differences for genes encoding Ig have been demonstrated (Walther et al., 2015), which could explain why purebred animals or some specific horse breeds have higher IgG values, either because their basal levels are higher, or because their immune system responds more severely to the infection.

Another risk factor found in this study was the use of horses, with higher seroprevalence in horses used for breeding than in others. Although this factor has not been evaluated so far in any other study, life habits have been observed as a risk factor for *T. gondii* infection in previous studies. Specifically, Liang et al. (2022) found higher seroprevalence in horses in cage-free farms than in captive farms, and the seroprevalence was higher in farms than in equestrian centres (Ouslimani et al., 2019). Farm location has also been demonstrated as a risk factor, given that the seroprevalence is higher in rural herds than in urban herds (Alvarado-Esquivel et al., 2012). All the animals included in our study lived in rural equestrian centres, so we were unable to verify this risk factor in our study. In as much as the horses used for breeding were usually living in cage-free rural farms, this could explain the higher seroprevalence in animals used for breeding.

Effects on sex and age have contradictory results in the bibliography. In line with our results, studies in Tunisia (Boughattas et al., 2011) and Turkey (Karatepe et al., 2010) did not observe statistical difference between males and females, whereas Marzok et al. (2023) found high seroprevalence in mares. These authors also found high seroprevalence in elder animals, in contrast to our work. Published data regarding the effect of age on the seroprevalence of *T. gondii* infection are contradictory. While some authors found high seroprevalence in young horses (Kouam et al., 2010; Marzok et al., 2023), others observed low seroprevalence in these animals (Zhang et al., 2018). Finally, several studies, including the present one, indicate that there is no effect of age on seroprevalence of *T. gondii* in horses (Alvarado-Esquivel et al., 2012; García-Bocanegra et al., 2012; Ouslimani et al., 2019).

5. Conclusions

In conclusion, the prevalence of *T. gondii* infection in horses differs between detection methods, being 11.43% by serological method (ELISA) and 27.62% by molecular methods. Purebred animals and horses used for breeding present higher seroprevalence, but no other risk factors were found. Horse meat is commonly consumed in several countries in the Mediterranean region, and the high seroprevalences observed indicate that this species could be a reservoir of the parasite, in addition to a possible risk to public health. Analysis by serological and/or molecular methods in blood samples of horses could be an effective and rapid method for detecting *T. gondii* infection before the meat reaches the consumer. Further studies to analyse the correlation between seroprevalence and the presence of parasite DNA in blood with the presence of transmitting cysts in meat are necessary to determine the extent of the risk associated with horse meat consumption, especially in countries of greater production like Spain.

Ethics approval and consent to participate

The experimental protocol was approved by the Ethics Committee of Universidad Cardenal Herrera CEU (protocol code VCS/022/PEA/0279). The experimental protocols complied with the guidelines of the Ethic Committee. Blood samples were collected by the researchers (veterinarians) after getting informed consent from the owners of the horses. All efforts were made to minimize animal suffering during sample collection. Oral and written informed consent was obtained from all people who participated in the study.

Funding

This research was funded by Universidad Cardenal Herrera, grant numbers IDOC23–01, INDI23–35 and GIR23–35. Lola Martínez-Sáez are supported by a Predoctoral Contract form the Universidad Cardenal Herrera CEU. The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication.

CRediT authorship contribution statement

Lola Martínez-Sáez: Writing – original draft, Methodology, Data curation. **Samuele Pala:** Writing – original draft, Methodology, Data curation. **Pablo Jesús Marín-García:** Formal analysis. **Lola Llobat:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgements

We are grateful to the University Cardenal Herrera CEU.

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