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SHORT COMMUNICATION

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Seroepidemiological study of *Toxoplasma gondii* in equids in different European countries

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Abstract

Toxoplasmosis, caused by the obligate intracellular protozoan *Toxoplasma gondii*, is a worldwide parasitic zoonosis. A cross-sectional study was carried out to determine the exposure to *T. gondii* in equids in Europe. Serum samples from 1399 equids (1085 horses, 238 donkeys, and 76 mules/hinnies) bred in four European countries (Italy, Spain, United Kingdom [UK], and Ireland) were collected during the period of 2013–2021. The overall seroprevalence of *T. gondii* was 18.9% (95% Confidence Interval [CI]: 16.9–21.0) by using the modified agglutination test (MAT) at a cut-off of 1:25. Seropositivity by country was 27.1% in Italy, 16.6% in Spain, 12.0% in UK and 7.0% in Ireland. Anti-*T. gondii* antibodies were detected in 12.8% of the horses, 43.7% of the donkeys, and in 28.9% of the mules/hinnies. A Generalized Estimating Equation (GEE) analysis was carried out to study the associations between seropositivity and explanatory variables related to individuals, herds, and management measures on these herds, selected based on the bivariate analysis. The risk for being seropositive for *T. gondii* was 5.3 and 2.7 times higher in donkeys and mules/hinnies than in horses, respectively. In addition, significantly higher seropositivity was observed in horses

from herds that used disinfectants less than once a week (13.9%; $p = 0.038$, odds ratio [OR] = 1.6; 95% CI: 1.03–2.62) compared with those from herds that performed weekly disinfection of the facilities (9.4%). This is the first large-scale seroepidemiological study on *T. gondii* comprising horses, donkeys, and mules/hinnies in Europe and the first report of *T. gondii* exposure in horses from Ireland and UK. We found a widespread distribution of *T. gondii* among equid populations in different European countries. The seroprevalence found in these species, especially in donkeys and mules/hinnies, highlights the potential risk of human infection through the consumption of their raw/undercooked milk or meat.

KEYWORDS
equids, food-borne pathogens, toxoplasmosis, zoonosis

1 | INTRODUCTION

Toxoplasmosis is a worldwide parasitic zoonosis caused by *Toxoplasma gondii*. Its sexual reproduction occurs in felid (definitive) hosts, which excrete oocysts in faeces, and involves virtually all homeotherm species as intermediate hosts (Dubey, 2022). Humans or animals typically become infected by three main modes of transmission: consumption of tissue cysts from infected raw or undercooked meat, ingestion of food or water contaminated with sporulated *T. gondii* oocysts and by transplacental or blood transmission with tachyzoites. *Toxoplasma gondii* infection is usually sub-clinical in immunocompetent individuals, however, this opportunistic pathogen can lead to abortion, stillbirth, and congenital abnormalities as well as serious disease and even death in immunocompromised people (Dubey, 2022). Moreover, the relationship between *T. gondii* latent infections and chronic neurological and psychiatric conditions (mainly schizophrenia and bipolar disorder) has been previously suggested (Fabiani et al., 2015; Fuglewicz et al., 2017; Milne et al., 2020).

Equids were domesticated 5000–6000 years ago, and since then, they have been used by humans for different purposes including transportation of people and goods, hunting, war, farming or leisure. In addition, equids have also served as a food source since the late Palaeolithic (50,000–12,000 before the present [B.P.]) (Li et al., 2020; Stanciu, 2015). Currently, equine meat is still consumed in many countries, and in the European Union (EU) alone, the average consumption is approximately 110,000 tons per year (FAO, 2013).

Viable *T. gondii* cysts has been isolated by mouse bioassay from horse and donkey meat (Al-Khalidi & Dubey, 1979; Dubey et al., 2020; Evers et al., 2013; Paştiu et al., 2015) and clinical toxoplasmosis has been epidemiologically related with horse meat consumption in humans (Pomares et al., 2011). Also, donkey milk consumption is becoming progressively more popular in recent years, and it has been suggested that human consumption of raw milk from seropositive donkeys could be a potential source of toxoplasmosis (Mancianti et al., 2014). In addition, although scientific evidence indicates that horses and other equids are among the most resistant domestic species to clinical toxoplasmosis (Dubey, 2022), the association between *T. gondii* seropositivity and clinical equine protozoal myeloencephalitis in horses has also been suggested (James et al., 2017; Schale et al., 2018). Recently, fatal toxoplasmosis was diagnosed in a horse in the USA (Kimble et al., 2021).

In farm animals, including equids, serological tests are the first-line strategies for diagnosis of *T. gondii*, while PCR methods are specific, sensitive, and useful for detection of parasite DNA in animal tissues (Almeria & Dubey, 2021). Indirect diagnosis methods have few drawbacks because antibody detection only indicates exposure to the parasite and not the presence of active infection. However, serological tests are useful tools for survey studies since they allow to identify *Toxoplasma*-positive farms and animals as well as to analyse the related risk factors associated with the exposure to the parasite (Dubey, 2022). Several serological studies on *T. gondii* exposure in equids have been carried out worldwide (reviewed in Dubey et al., 2020). However, there is no information about *T. gondii* seroprevalence in equids in many European countries and updated information in these species is scarce in others. The aims of the present study were to determine the prevalence of *T. gondii* antibodies and to identify risk factors associated with the exposure to this zoonotic protozoan in equids in different European countries.

Impacts

- *Toxoplasma gondii* is widespread in equid populations in different European countries.
- Donkeys and mules/hinnies are at the highest species risk for *T. gondii* positivity.
- Raw or undercooked foods from equids could be of public health concern in many European regions.

2 | MATERIALS AND METHODS

2.1 | Sampling

Blood samples from 1399 equines were collected in southern/north-eastern Spain (n = 757 from 143 municipalities), south-central Italy (n = 442 from 36 municipalities), southeastern UK (n = 100 from three municipalities) and Ireland (n = 100 from 23 municipalities) during the period 2013–2021.

Two equid species, horses (n = 1085) and donkeys (n = 238), and two equid hybrids mules/hinnies (n = 76), were sampled (Figure 1). Mules are a cross between a female horse and a male donkey, and hinnies are a cross between a female donkey and a male horse. Samples from Spain and Italy were obtained from serum banks of horses sampled in epidemiological surveillance programs or submitted to medical check-ups during the study period. Donkeys and mules/hinnies were also included from these sources when possible. More than 385 animals, that provides the highest sample size with a CI95% and accepted error of 5% in studies based on unknown prevalence assuming a prevalence of 50% (Thrusfield, 2018), were sampled in each country. In addition, a convenience sampling was also carried out in Ireland and the UK from equids subject to surgical interventions or medical check-ups during the study period in veterinary hospitals. Samples were obtained by puncture of a jugular vein and sera were obtained by centrifugation at 400g for 10 min and were kept frozen at −20°C until ready to be sent to the Animal Health Department at the University of Cordoba (Spain) for serological analysis.

Whenever possible, individual and herd epidemiological information was gathered from each animal (Table 1).

2.2 | Serological analysis

Presence of *T. gondii* antibodies in the sera from equids was assayed by the modified agglutination test (MAT) following the protocol described by Dubey and Desmonts (1987). A titre of 1:25, which is recognized as highly sensitive and specific for detection of *T. gondii* antibodies, was used as a cut-off for *T. gondii* seropositivity as previously considered for these species (Dubey et al., 2020). All positive sera were retested and titrated at dilutions: 1:25, 1:50, 1:100 and 1:500. Although there is no validation of MAT for the detection of *T. gondii* in equids, extensive studies in chickens, pigs, and sheep attest the validity of MAT for the diagnosis of toxoplasmosis in animals (reviewed in Dubey, 2022).

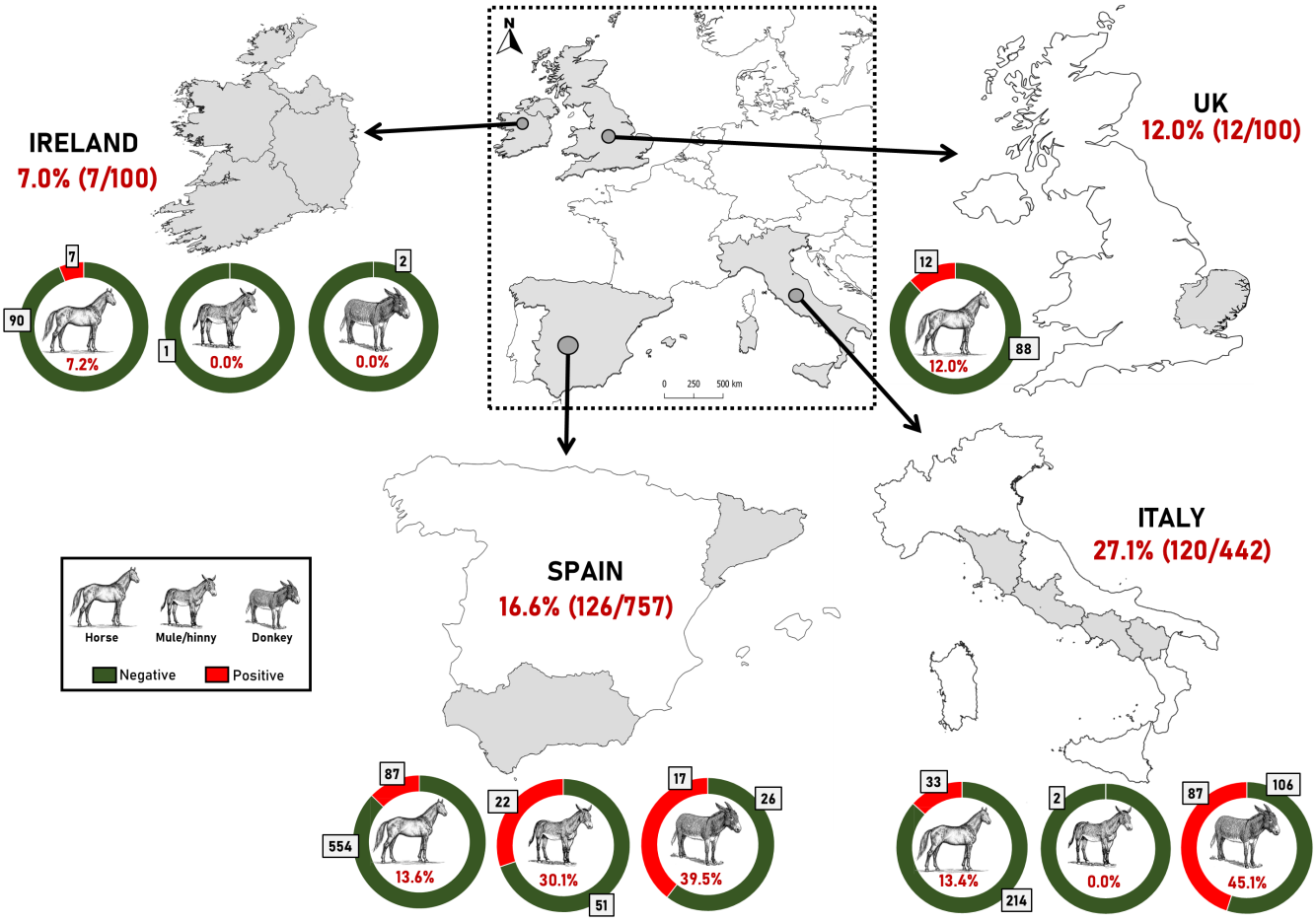

FIGURE 1 Seroprevalence of *Toxoplasma gondii* by equid species and country.

TABLE 1 Distribution of the seroprevalence of *Toxoplasma gondii* in equids and horses by categories and results of the bivariate analysis

		EQUIDS			HORSES		
Variable	**Categories**	**% MAT positive**	**Positive/overall[a]**	***p-value***	**% MAT positive**	**Number/overall[a]**	***p-value***
Species	Horse	12.8	139/1085				
	Donkey	43.7	104/238	<0.001			
	Mule/hinnie	28.9	22/76				
Sex	Male	13.8	76/552	<0.001	11.4	56/491	0.332
	Female	22.7	161/710		12.5	58/463	
Country	Ireland	7.0	7/100		7.2	7/97	
	Italy	27.1	120/442		13.4	33/247	
	United Kingdom	12.0	12/100	<0.001	12.0	12/100	0.365
	Spain	16.6	126/757		13.6	87/641	
Age	Foal	18.4	49/266		10.3	19/185	
	Adult	19.9	128/643	0.730	11.7	55/470	0.360
	Geriatric	18.0	65/361		14.3	44/307	
Breed	Mixed-breed	15.4	44/285	0.024	13.6	35/258	0.204
	Purebred	21.0	174/830		11.3	63/559	
Activity	Sport	12.3	40/325		12.4	40/322	
	Milk production	44.7	80/179		–	–	
	Work	18.8	9/48	<0.001	9.1	2/22	0.972
	Breeder	22.5	50/222		12.3	18/146	
	Family leisure	13.2	37/281		11.9	30/252	
Presence of cats	No	25.4	106/417	0.030	16.0	46/288	0.121
	Yes	19.9	93/467		12.3	38/309	
Census of equids	>60	21.9	57/260		9.8	16/164	
	14–60	24.9	67/269	0.136	10.8	17/158	0.084
	<14	18.0	51/284		16.5	42/255	
Shelter (spring–summer)	Indoor/Outdoor	14.2	16/113		9.9	9/91	
	Outdoor	19.8	113/570	0.107	12.5	52/417	0.610
	Indoor	23.4	69/295		14.0	29/207	
Shelter (autumn-winter)	Indoor/Outdoor	17.4	16/92		14.1	11/78	
	Outdoor	21.3	80/375	0.703	11.4	27/236	0.778
	Indoor	20.7	106/511		13.0	49/377	
Cleaning of facilities	Occasionally	25.6	126/492	<0.001	9.5	23/242	0.045
	Weekly	15.6	77/493		14.3	65/456	
Disinfection of facilities	Occasionally	22.9	173/756	0.001	13.9	70/505	0.069
	Weekly	13.2	30/228		9.4	18/192	
Rodent control	No	22.6	145/642	0.016	11.9	49/411	0.354
	Yes	16.6	56/337		13.2	37/281	
Well water to drink	No	19.9	185/931	0.068	12.6	86/682	0.418
	Yes	15.4	37/240		11.7	24/205	
Water from ponds and streams to drink	No	18.3	199/1088	0.028	12.1	103/848	0.199
	Yes	27.7	23/83		17.9	7/39	
Tap water to drink	No	15.1	97/644	<0.001	12.6	73/578	0.434
	Yes	23.7	125/527		12.0	37/309	
Reproductive disorders in the last year	No	22.1	145/656	0.521	11.7	51/435	0.586
	Yes	14.3	1/7		14.3	1/7	

[a]Missing values omitted.

2.3 | Statistical analysis

The overall prevalence of antibodies was estimated from the ratio of positives to the total number of analysed samples, with a 95% confidence interval (CI). The continuous variables were categorized considering percentiles 33 and 66 as cut-off points. Associations between explanatory variables and seropositivity for *T. gondii* were tested in three steps. First, a bivariate Pearson's chi-square test or Fisher's exact test, as appropriate, was performed to obtain the relevance of the explanatory variables in the risk of an animal being *T. gondii* seropositive. Variables that showed biological plausibility and with $p \leq 0.10$ in the bivariate analysis were selected as potential risk factors. Secondly, collinearity between pairs of variables was tested by Cramer's V coefficient. When collinearity between variables was detected ($p < 0.05$), only the variable more clearly linked to *T. gondii* from an epidemiological point of view was retained. Finally, a multivariable Generalized Estimating Equations (GEE) model was carried out to study the effect of potential explanatory variables previously selected in the bivariate analysis (Hanley et al., 2003). The number of seropositive animals was assumed to follow a binomial distribution and 'municipalities' was defined as the subject variable. A Poisson error distribution and a logarithmic link function were considered. Biologically plausible confounding factors were tested using Mantel–Haenszel analysis and confounding was considered to be potentially significant if odd ratios shifted appreciably. Changes in the OR greater than 30% were considered indicative of confounding. Potential two-way interactions between the variables were tested for significance in the model. The multivariable model was re-run until all remaining variables presented statistically significant values ($p < 0.05$), and potential causal relationship with the response variable existed. The choice of the best model was based on the Quasi-likelihood under independence model criterion (QIC). Statistical analyses were performed using SPSS 25.0 software (IBM Corp.).

3 | RESULTS

Anti-*T. gondii* antibodies were detected in 265 of 1399 equids (18.9%; 95% CI: 16.9–21.0). Seropositivity was found in 12.8%, 43.7%, and 28.9% of the 1085 horses, 238 donkeys and 76 mules/hinnies analysed, respectively (Figure 1, Table 1). *Toxoplasma gondii* titres observed were 1:25 in 50.9%, 1:50 in 24.2%, 1:100 in 23.8% and ≥1:500 in 1.1% of the tested samples. At least one seropositive animal was detected in 80 of 205 (39.0%) municipalities sampled.

Table 1 shows the distribution of the seroprevalence of *T. gondii* in equids and horses by the explanatory variables obtained from the epidemiological questionnaire and results of the bivariate analysis.

A total of 8 out of 17 explanatory variables associated ($p < 0.10$) with *T. gondii* seropositivity in equids were selected after data exploration and bivariate analyses and included in the multivariable analysis. The final GEE multivariable analysis showed the species as a risk factor potentially associated with the exposure to *T. gondii* (Table 2). Significantly higher seropositivity was found in donkeys (43.7%; $p < 0.001$, OR = 5.3; 95% CI: 3.65–7.57) and mule/hinnies (28.9%; $p < 0.001$, OR = 2.7; 95% CI: 1.79–4.15) compared with horses (12.8%).

To avoid potential bias associated to co-linearity, horses, the species with the most homogeneous sampling among countries, were statistically assessed separately. In this species, three variables ('census of equids', 'cleaning of facilities' and 'disinfection of facilities') were selected from the bivariate analyses ($p \leq 0.10$) (Table 1), and the final GEE model identified the 'occasionally disinfection' of facilities (less than once a week) as the only risk factor associated with *T. gondii* exposure in horses (Table 2). Significantly higher seropositivity was observed in horses from herds that used disinfectants <1 week (13.9%; $p = 0.038$, OR = 1.6; 95% CI: 1.03–2.62) compared with those from herds that performed weekly disinfection of the facilities (9.4%).

4 | DISCUSSION

The present study is the first large-scale seroepidemiological study on *T. gondii* comprising horses, donkeys, and mules/hinnies in Europe, and to the best of the authors' knowledge, this is also the first report of *T. gondii* exposure in equids from the UK and Ireland. The results indicate a wide exposure of *T. gondii* in equid populations in the European countries analysed.

TABLE 2 Results of the generalized estimating equations model of potential risk factors associated with *T. gondii* seropositivity in equines and horses

Variable	Categories	β	*p*-value	OR	95% CI
	EQUIDS				
Species	Horse	[a]	[a]	[a]	[a]
	Donkey	1.66	<0.001	5.26	3.65–7.57
	Mule/hinnie	1.00	<0.001	2.72	1.79–4.15
	HORSES				
Disinfection of facilities	Weekly	[a]	[a]	[a]	[a]
	Occasionally	0.49	0.038	1.64	1.03–2.62

[a]Reference category.

Wide fluctuations in *T. gondii* seropositivity have been reported among equids from different countries and continents (Dubey et al., 2020). The overall seroprevalence observed in horses in the present study (12.8% of 1085) is within the range previously reported in Europe (Dubey et al., 2020). Similar antibody prevalences against *T. gondii* were found in this species in Spain (10.8% of 454) (García-Bocanegra et al., 2012) and Portugal (13.3% of 173) (Lopes et al., 2013). Lower *T. gondii* seroprevalence values were detected in Greece (1.7% of 753) (Kouam et al., 2010), Italy (3.0% of 643) (Bártová et al., 2015) and Ukraine (7.9% of 76) (Rissanen et al., 2019). Conversely, higher seropositivity rates were detected in Italy (17.6% of 153) (Papini et al., 2015), Czech Republic (24.0% of 552) (Bártová et al., 2010), Romania (37.8% of 82) (Paştiu et al., 2015), and France (58.9% of 231) (Aroussi et al., 2015). Comparing and interpreting results obtained from equines using different study designs, serological methods, and cut-offs to define seropositivity is challenging, and comparisons should be carefully made. In this respect, the survey study carried out by García-Bocanegra et al. (2012) in southern Spain, in 2011, was performed using the same serological technique and cut-off values, and results could, therefore, be compared. The similar seropositivity observed in horses in the present study in the same area (14.2%; 36/254) suggest an endemic circulation of *T. gondii* in this region during the last decade.

The information on *T. gondii* exposure in donkeys and mules/hinnies in Europe is still very limited. In the present study, the overall seroprevalence of *T. gondii* in donkeys (43.7% of 238) represents the highest found in this species in Europe to date. Lower seroprevalence values were observed in two previous studies carried out in the Iberian Peninsula: 5.9% (11/186) (Rodrigues et al., 2019) and 25.6% (21/82) (García-Bocanegra et al., 2012), and in Italy: 25.0% (11/44) and 5.0% (12/238) (Machacova et al., 2014; Mancianti et al., 2014). Regarding mules/hinnies, there have been very few serological studies of *T. gondii* reported worldwide to date. Lower seroprevalence rates to those found in the present study (28.9% of 76) were reported in the two previous serosurveys performed in this species in Europe. Kouam et al. (2010) detected 7.6% of anti- *T. gondii* antibodies in 13 mules from Greece, while the seropositivity observed by García-Bocanegra et al. (2012) in this species in Spain was 15.0% (12/80).

The risk of *T. gondii* seropositivity was 5.3 and 2.7 times higher in donkeys and mules/hinnies than in horses. The higher seropositivity detected in donkeys, followed by mules/hinnies and horses, are in accordance with those previously reported (de Oliveira et al., 2013; García-Bocanegra et al., 2012; Kouam et al., 2010; Munhoz et al., 2019). Differences in farming systems among equids could explain the differences observed in this study. In this regard, donkeys and mules are usually raised under extensive management conditions, increasing the risk of seropositivity to *T. gondii* by ingesting oocysts contaminating the environment (García-Bocanegra et al., 2012). Moreover, Munhoz et al. (2019) suggested that donkeys and mules could maintain detectable *T. gondii* antibodies titers for longer than horses. In any case, further studies are needed to assess differences in the susceptibility to infection among equid species.

Due to the high seroprevalence detected in donkeys in the present study, the risk associated with donkey raw milk consumption should also be considered, especially taking into account that in the last decade, due to its properties, donkey milk has become very popular in industrialized countries as a food source (Dubey et al., 2014; Machacova et al., 2014; Mancianti et al., 2014; Martini et al., 2014).

When only horses were assessed in the statistical analyses, the occasional disinfection of facilities (less than once a week) was shown to be a risk factor associated with *T. gondii* seropositivity. Accordingly, it has been widely suggested that a good hygienic status of the farm and the implementation of proper disinfection measures has a protective effect against *T. gondii* infection in livestock (reviewed in Stelzer et al., 2019).

Of note, statistically significant differences were not found in the seroprevalence of *T. gondii* in horses among countries ($p = 0.365$) (Table 1). This result could indicate that weather conditions did not play a major role in *T. gondii* seropositivity in horses. In this regard, in Spain, where a wider sampling was carried out, statistical differences ($p = 0.402$) between the south (14.2% of 254 horses) and the northeast (13.2% of 387 horses) of the country were also not observed despite the weather differences. In any case, further studies, increasing the number of animals sampled, are warranted to support this hypothesis.

This study has some limitations. First, given the limited number of samples in Ireland and the UK, extrapolating the results to the entire equine population of these countries should be carefully made. In addition, due to the lower census of donkeys and mules/hinnies, there is a great variation in the number of individuals of the studied animal species. Finally, disinfectants used on the studied herds could not be individually discriminated. Future studies are warranted including a larger number of equids (especially donkeys and mules/hinnies) to verify results of the present investigation. Moreover, given *T. gondii* oocysts are resistant to most used disinfectants (CFSPH, 2017; Dubey, 2022), newer compounds are needed to implement appropriate control measures.

The seroprevalence values detected in the present study indicate a widespread distribution of *T. gondii* among equid populations in different European countries, with donkeys and mules/hinnies being the species at highest risk of *T. gondii* exposure. Given the high seroprevalences found in the present study, raw or undercooked foods from these species could be of public health concern in many European regions. Boiling of milk and an adequate cooking of meat (considering that tissue cysts remain viable for approximately 4 min at 60°C) could be suitable measures to prevent zoonotic infection by this parasite (CFSPH, 2017). In addition, a frequency of disinfection of the facilities of at least once a week could reduce the risk of *T. gondii* seropositivity in horse herds.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the authors upon reasonable request.

ETHICS STATEMENT

Horse samples were obtained within the official Epidemiological Surveillance System Programs and from specimens subjected to medical check-ups or surgical interventions during the study period. Animal handling and sampling were performed by qualified and trained veterinarians following European (Directive 86/609/CEE). Therefore, no ethical approval was necessary.

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