

Research Article

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Seroprevalence of antibodies of *Neospora* spp. and *Toxoplasma gondii* in horses from southern Italy

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Abstract: The consumption of horse meat has been epidemiologically linked to clinical toxoplasmosis in humans and neosporosis that may cause clinical illness in horses. Here we determined seroprevalence of antibodies against *Toxoplasma gondii* Nicolle et Manceaux, 1908 and species of *Neospora* Dubey, Carpenter, Speer, Topper et Uggla, 1988 in horses from Italy. Blood samples were collected from 643 apparently healthy horses from 60 farms of 51 municipalities in southern Italy. The presence of antibodies against *T. gondii* and *Neospora* spp. were detected by indirect fluorescence antibody test (IFAT); a titre ≥ 50 was considered positive. The same sera were also tested for antibodies against *Neospora* spp. by a competitive-inhibition enzyme-linked immunosorbent assay (cELISA); samples with $\geq 30\%$ inhibition were considered positive. Antibodies against *T. gondii* and *Neospora* spp. were detected in 19 (3.0%) and 15 (2.3%) horses by IFAT, respectively, without statistical difference between gender, age and breeds (p -value ≥ 0.05). Antibodies against species of *Neospora* were detected in 70 (10.9%) horses by cELISA with statistical difference in gender (6.0–18.5%, p -value ≥ 0.05) and breeds (0–19.4%, p -value ≥ 0.05). Although *T. gondii* infection rates were low, the risk of human infection should not be dismissed, particularly in Italy where consumption of raw or undercooked horse meat has a long tradition.

Keywords: serological test, *Equus caballus*, risk factor, toxoplasmosis, neosporosis

Toxoplasma gondii Nicolle et Manceaux, 1908 and *Neospora* Dubey, Carpenter, Speer, Topper et Uggla, 1988 are related coccidians and important pathogens (Dubey and Lindsay 1996, Dubey 2010). Severe clinical toxoplasmosis in France was epidemiologically linked to the ingestion of uncooked horse meat (Pomares et al. 2011) and viable *T. gondii* has been isolated from horses slaughtered for human consumption (Dubey 2010). Since undercooked horse meat is known to be consumed by people in several developed countries including Italy, we determined seroprevalence of *T. gondii* in horses from southern Italy. Neosporosis can cause clinical illness in horses (Kligler et al. 2007) but few data are available on its occurrence in Italy. Therefore, we also detected antibodies against this apicomplexan in the same horses from southern Italy.

MATERIALS AND METHODS

Between September–December 2013, blood samples were collected by venipuncture from 643 horses appearing healthy, raised on 60 farms of 51 municipalities in southern Italy. This sample

size was calculated using the formula advocated by Thrusfield (2007) inserting the following values: study population in south Italy (75 263 horses, data supplied by the Italian Association of Breeders 2013), expected prevalence of toxoplasmosis (20%, data reviewed in horses tested in Italy; Tassi 2007), confidence interval (99%), and desired absolute precision (5%). The horse owners participated voluntarily in this study and background data on animals were obtained through a questionnaire filled during sample collection (Table 1).

Blood samples of horses were collected from a jugular vein using a vacuum tube without anticoagulant. Blood was centrifuged; serum was removed and stored at -20°C . The presence of antibodies against *Toxoplasma gondii* and *Neospora* spp. were detected by indirect fluorescence antibody test (IFAT) using commercially available IFR antigens of *T. gondii* and *Neospora caninum* Dubey, Carpenter, Speer, Topper et Uggla, 1988 (VMRD, Pullman, Chicago, USA), respectively, and anti-horse IgG FITC conjugate (VMRD). The sera were diluted with physiological solution two-fold starting at titre 1 : 50; a titre of 50 was considered positive for both tests. Procedure in brief: antigen of *T. gondii*

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Table 1. Characteristics of horses and their seroprevalence to antibodies of *Toxoplasma gondii* Nicolle et Manceaux, 1908 and species of *Neospora* spp.

Characteristics	Horses tested	<i>Neospora</i> spp.			<i>Toxoplasma gondii</i>	
		IFAT positive (%)	cELISA positive (%)	<i>p</i> -value ^a	IFAT positive (%)	<i>p</i> -value ^a
Gender						
Female	315	5 (1.6%)	32 (10.2%)	IFAT (<i>p</i> = 0.434), cELISA (<i>p</i> = 0.0013)	7 (2.2%)	<i>p</i> = 0.5472
Male	182	5 (2.8%)	11 (6.0%)		7 (3.9%)	
Castrate	146	5 (3.4%)	27 (18.5%)		5 (3.4%)	
Age categories (years)						
< 1–4	105	3 (2.9%)	10 (9.5%)	IFAT (<i>p</i> = 0.4507), cELISA (<i>p</i> = 0.7281)	3 (2.9%)	<i>p</i> = 0.8062
≥ 4–9.5	196	3 (1.5%)	27 (13.8%)		4 (2.0%)	
≥ 9.5–15	178	4 (2.3%)	20 (11.3%)		6 (3.4%)	
≥ 15–34	111	5 (4.5%)	13 (11.7%)		2 (1.8%)	
not known	53	0	0		4 (7.6%)	
Breed*						
Appaloosa	34	1 (2.9%)	5 (14.7%)	IFAT (<i>p</i> = 0.3541), cELISA (<i>p</i> = 0.0015)	0	<i>p</i> = 0.2984
Pony	59	3 (5.1%)	6 (10.0%)		0	
Quarter Horse	62	2 (3.2%)	12 (19.4%)		2 (3.2%)	
Salernitano	30	0	0		1 (3.3%)	
Sella Italiana	92	2 (2.2%)	3 (3.3%)		6 (6.5%)	
Trotters	71	0	2 (2.8%)		2 (2.8%)	
crossbreed	60	0	7 (11.7%)		1 (1.7%)	
other breeds	111	5 (4.5%)	18 (16.2%)		2 (1.8%)	
not known	124	2 (1.6%)	17 (13.7%)		5 (4.0%)	
Total	643	15 (2.3%)	70 (10.9%)			

IFAT – indirect fluorescence antibody test; cELISA – competitive-inhibition enzyme-linked immunosorbent assay; *other breeds – 23 different breeds with number of animals ≤ 20; ^a *p*-value ≤ 0.05 statistically significant difference.

or *N. caninum* fixed on glass slide was overlaid with 15 µl of the examined serum and incubated in a humid chamber 30 min at 37°C followed by washing (2 × 10 min), drying and applying 15 µl of specific conjugate. Then, the slides were incubated 30 min at 37°C in a humid chamber. After washing (2 × 10 min) and drying, the smear was overlaid with 80% glycerol (pH 7.4) and covered with cover glass and the smears were examined with fluorescence microscope OLYMPUS BX 41 at 1000× magnification with oil immersion. Continuous peripheral fluorescence was considered specific. Sera from horse screened by latex agglutination test and IFAT served as *T. gondii*- and *N. caninum*-positive and negative controls that were included in each slide.

Neospora spp. antibodies were also detected by competitive-inhibition enzyme-linked immunosorbent assay (cELISA, VMRD). Serological examination and evaluation was done according to the manufacturer's instructions; samples associated with percent inhibition value ≥ 30% were considered positive. The optical density values were obtained using an automatic plate reader (Dynex Technology MRXII, Prague, Czech Republic).

Seroprevalence was statistically analysed, considering the variables of gender, age and breeds. The data analysis was performed by Chi-Square test for independence or rather Fisher exact test for tables 2 × 2 using (StatSoft, Inc. 2013). Dependence of age and seroprevalence was evaluated by Wilcoxon test. We tested null hypothesis that *T. gondii* and *Neospora* spp. seroprevalences do not depend on gender, age and breeds. The differences were considered statistically significant when *p*-value was ≤ 0.05. Horses from groups with unknown age or breed were not included in statistic analysis.

RESULTS

Antibodies against *Toxoplasma gondii* and *Neospora* spp. were detected by IFAT in 19 (3.0%) and 15 (2.3%)

of 643 horses, respectively, with titres ranging from 1 : 50 to 1 : 100 for *T. gondii* and 1 : 50 for *Neospora* spp. (Table 1). Antibodies against *Neospora* spp. were tested also by cELISA with positive inhibition in 70 (10.9%) horses. Co-infection with *T. gondii* and *Neospora* spp. was detected in two horses (0.3%).

Higher *T. gondii* seroprevalence was detected in males (3.9%) compared to castrates (3.4%) and females (2.2%), in age category ≥ 9.5–15 years (3.4%) and Sella Italiana breed (6.5%) compared to the other groups. However, differences of seroprevalence with *T. gondii* were not statistically significant (*p*-value ≥ 0.05) between gender, age and breed categories of horses (Table 1).

Higher seroprevalence with *Neospora* spp. was detected by IFAT in castrates (3.4%) compared to males (2.8%) and females (1.6%), in age category ≥ 15–34 (4.5%) and breed Pony (5.1%) compared to the others groups, but without statistical difference (*p*-value ≥ 0.05). In cELISA, statistical difference was found for seroprevalence with *Neospora* spp. between gender [test statistics = 13.28, degrees of freedom (df) = 2, *p*-value = 0.0013] and breeds (test statistics = 23.40, df = 7, *p*-value = 0.0015). Higher prevalence of *Neospora* spp. was found in castrates (18.5%) compared to males (6.0%) and females (10.2%) and in Quarter Horse breed (19.4%) compared to the other breeds (0–16.2%).

DISCUSSION

In the present study, antibodies against *T. gondii* were found in 3.0% of 643 horses by IFAT. In Europe, there are similar studies in horses, but using different serological methods (Table 2). In our study, we did not find statistically different seroprevalence of *T. gondii* between gender, age and breeds of horses, but the overall prevalence was low. In

Table 2. Seroprevalence of antibodies to *Toxoplasma gondii* Nicolle et Manceaux, 1908 in horses from Europe.

Country	No. of horses	Prevalence (%)	Method	References
Czech Republic	552	23	LAT	Bártová et al. 2010
Greece	773	2	ELISA	Kouam et al. 2010
Italy	163	31	MAT	Tassi 2007
	643	3	IFAT	Present study
Netherlands	85	7	ELISA	Knapen et al. 1982
Spain	454	11	MAT	Garcia-Bocanegra et al. 2012
Switzerland	120	4	ELISA	Wyss et al. 2000
Turkey	168	37	SFR	Gazayagci et al. 2011

LAT – latex agglutination test; ELISA – enzyme-linked immunosorbent assay; IFAT – indirect fluorescent antibodies test; MAT – modified agglutination test; SFR – Sabin-Feldman reaction.

Spain, Garcia-Bocanegra et al. (2012) found statistical different prevalence in crossbreed compared to pure breeds, but other factors such as age, gender and keeping horses inside or outside or presence of cats on farms were not found to have effect on prevalence of *T. gondii*. In Greece, the type of activity and location has been found to have a significant effect on the presence of *T. gondii* in horses (Kouam et al. 2010).

Currently, there is no evidence that *T. gondii* causes clinical disease in naturally or experimentally infected horses but viable *T. gondii* has been isolated from naturally exposed horses (Dubey 2010). Pomares et al. (2011) described a case of severe clinical toxoplasmosis from man in France who ingested uncooked imported horse meat. This indicates that consumption of horse meat may be a potential source of human infection. Horses used for human consumption are mainly working animals, riding or racing horses that are not used anymore and they are slaughtered. Horses from our study could be taken as a representative group for detection of possible danger of *T. gondii* for human consumption in Italy.

In the present study, we found antibodies against *Neospora* spp. in 2.3% and 10.9% of 643 horses by IFAT and cELISA, respectively. In Europe, antibodies against *Neospora* spp. were detected in 0.4% healthy horses from the Czech Republic (Bártová et al. 2010), 6% in France (Pitel et al. 2003), 9% horses from Turkey (Kilbas et al. 2008) and 9% in Sweden (Jakubek et al. 2006). Similarly, as in the case of infection with *T. gondii*, we detected much lower prevalence of *Neospora* spp. compared to results of Piantedosi et al. (2009) and Ciaramella et al. (2004) who detected by IFAT seroprevalence of 9% and 28% in 297 and 150 horses from Italy, respectively. The IFAT is a subjective test because quantitation varies with the operator

reading the slides. The c-ELISA is a commercial standardised test and has been used for detecting antibodies against *Neospora* spp. in several hosts, including horses (Dubey et al. 2007).

Statistical difference was found in cELISA for seroprevalence with *Neospora* spp. between gender and breeds. Higher prevalence of *Neospora* spp. was found in castrates (18.5%) compared to males (6.0%). This could be explained by different management methods. Castrates in contrast to males are allowed to graze during the whole year together with other horses. On the pastures, they are in higher risk to be infected by oocysts that are spread to the environment in faeces excreted by dogs. Different seroprevalence of *Neospora* spp. in breeds was recorded in horses from USA (Pusterla et al. 2014), similarly as in horses from our study. However, there is no study explaining different sensitivity of breeds or crossbreeds to infections.

The ingestion of food and water contaminated with oocysts is considered the main route of infection with *T. gondii* and *Neospora* spp. because congenital toxoplasmosis has not been proven in horses and congenital neosporosis is infrequent. Data from our study also support this hypothesis. In the present study, horses from the youngest age group (eight horses in age from one month to one year) had no detectable antibodies to both *T. gondii* and *N. caninum*. Although seroprevalence with *T. gondii* is low in horses, the risk of human infection should not be dismissed mainly because a higher seroprevalence was found in older animals that are mostly used for human consumption.

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