



Short communication

Toxoplasma gondii in horse meat intended for human consumption in Romania



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ABSTRACT

The prevalence of *Toxoplasma gondii*, an economically important zoonotic protozoan, was investigated in horses slaughtered for export and human consumption in the North of Romania. Pairs of samples, sera and heart tissues, were collected from 82 slaughtered horses. Examination of horse sera by ELISA at a dilution of 1:10, and by modified agglutination test (MAT) at a dilution of 1:6, revealed that 32 (39%) and 31(37.8%) horses, respectively, had antibodies against *T. gondii*. Using polymerase chain reaction (PCR) analysis, *T. gondii* DNA was not found in any heart sample collected from horses. By bioassay in mice, we obtained viable isolates of *T. gondii* from two of ten horses determined to be strongly positive by serological assay/ELISA. The prevalence estimated in horses highlighted the potential risk for human contamination by consumption of raw or undercooked meat.

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1. Introduction

The seroprevalence of *Toxoplasma gondii* in naturally infected horses (*Equus ferus caballus*) worldwide, may range between 0% and 80% (Tassi, 2007) and consumption of raw or undercooked horse meat could be a potential source of human infection (Pomares et al., 2011).

In Romania, horses are primarily used for work and recreation, rather than for meat. Nevertheless, Romania (with Italy, Spain, Poland, Ireland, France, Germany, Belgium, the United Kingdom and the Netherlands), is among primary European exporters of horsemeat (Human Society International, 2014). Romanian horsemeat is consumed in Belgium, the Netherlands, Italy, Bulgaria and France (Human Society International, 2014). In 2011, Romania

exported 1378,800 kg of horsemeat to Belgium, 618,900 kg to the Netherlands, and 62,200 kg to France (Human Society International, 2012).

Although Romania exports horse meat, no study regarding the prevalence of *T. gondii* infection in this species was previously performed. In view of the lack of information regarding *T. gondii* infection in horse's meat, we aimed to determine the prevalence and the genotype of this parasite in slaughtered horses from Romania.

2. Materials and methods

2.1. Animals and serum

Paired samples of serum and heart tissues (the apex of the heart) were collected from 82 slaughtered horses. The weight of heart samples was between 57 and 72 g, the age of the animals varied between 1 and 22 years old (6.28 ± 6.17) and the carcass

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weight between 150 and 381 kg. Horses originated from backyard system, from Center and North–West of Romania, and were used for agriculture and transportation. Among the tested animals, 42 horses were previously diagnosed as serologically positive for equine infectious anemia virus (EIAV).

Blood was drawn from a jugular vein just prior to slaughter, and sera were transferred in Eppendorf tubes and kept in a freezer at -20°C until use. Also, the collected heart samples were stored at 4°C until use. IgG type antibodies against *T. gondii* were evaluated by ELISA and modified agglutination test (MAT). Then, based on serological results, tissue samples from seropositive horses were selected for bioassay in order to isolate and genotype the *T. gondii* strains. Moreover, all heart tissue samples were analyzed by polymerase chain reaction (PCR).

2.2. Enzyme-linked immunosorbent assay (ELISA)

T. gondii antibodies were detected by indirect ELISA, at a sera dilution of 1:10, using the commercial kit ID Screen Toxoplasmosis Indirect Multi-species (ID.vet; Innovative Diagnostics, Grabels, France) following the manufacturers' instructions. The results were expressed as S/P percentages according to the formula: $S/P\% = \text{OD sample} / \text{OD positive control} \times 100$. Sera with $S/P \leq 40\%$ were deemed as negative, between 40% and 50% doubtful, between 50% and 200% positive, and $\geq 200\%$ as strongly positive.

2.3. Modified agglutination test (MAT)

The modified agglutination test (MAT) for the detection of *T. gondii*-specific IgG antibodies was performed as previously described (Dubey and Desmonts, 1987), using an antigen prepared from formalin-fixed whole RH strain tachyzoites (Reims, France). Each serum sample was serially twofold diluted. The threshold dilution was 1:6 and the end dilutions of serum samples was 1:48.

2.4. Bioassay

We bioassayed in mice those heart samples from horses that were strongly seropositive as measured by ELISA. Briefly, each heart sample was ground up and mixed with 0.25% trypsin/0.025% EDTA solution, and then incubated for 2 h at 37°C . The homogenate was filtered through gauze, and then centrifuged. The supernatant was removed, and the pellet was resuspended in approximately 50 ml phosphate buffered saline (PBS, pH 7.2), and centrifuged again. We repeated this step three times to clean the trypsin solution. Finally, the pellet was resuspended in 3 ml PBS containing 100 μl antibiotic solution (20,000 units Penicillin/10,000 units Streptomycin). Two Swiss white mice per sample were each inoculated intraperitoneally with 0.5 ml of this suspension. Survivor mice were euthanized four weeks after inoculation, and their brains checked by microscope for the presence of *T. gondii* cysts. All mouse brains were analyzed by PCR for confirmation.

2.5. Deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from all heart samples ($n = 82$) collected from horses, and from the brains of inoculated mice ($n = 10$). For both type of tissues, we used the same protocol. We used a commercial kit (Isolate Genomic DNA kit; Bionline Reagents Limited, London, U.K.) following the manufacturer's protocol. DNA was extracted from 40 mg of tissue, and further tested for the presence of specific sequences of *T. gondii* by standard PCR, according to previously described protocols (Homan et al., 2000). We used *T. gondii* specific primers that amplify fragments of 529 bp: Tox 4 (5' CGCTGC AGG GAG GAA GAC GAA AGT TG 3'), and Tox 5 (5' CGCTGC AGA CAC

Table 1

Prevalence of anti-*Toxoplasma gondii* antibodies by ELISA in horses from Romania.

	No. animals	Frequency	Seroprevalence%	CI 95%
Males	44	20	45.5	24.36–54.5
Females	38	20	52.6	24.04–56.61
Young	21	6	28.6	11.28–52.18
Adult	40	18	45	29.26–61.51
Geriatric	21	8	38.1	18.11–61.56
EIAV positive	42	23	54.8	38.67–70.15
EIAV negative	40	9	22.5	10.84–38.5

AGT GCA TCT GGA TT 3') (Generi-Biotech, Hradec Králove, Czech Republic). PCR was carried out in a 25 μl reaction mixture consisting of 12.5 μl of MyTaq Red HS Mix (Bioline Reagents Limited) and 25 pM of each primer. The volume of DNA template was 4 μl . The amplification was performed in Bio-Rad C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules, California). Cycling conditions were: 1 min at 95°C ; 15 s at 95°C , 15 s at 60°C , and 10 s at 72°C (35 cycles); and 5 min at 72°C . Aliquots of each PCR product were electrophoresed on 2% agarose gel stained with SYBR[®] Safe DNA gel stain (Invitrogen, Carlsbad, California), and examined for the presence of the specific fragment under UV light (Bio-Rad BioDoc-ItTM Imagine System). DNA fragment size was compared with a standard molecular weight, 100 bp DNA ladder (Fermentas; Thermo Fisher Scientific, Waltham, Massachusetts). Two controls were performed: *T. gondii* RH strain was used as positive control, and distilled water was used as negative control.

3. Statistical analyses

Point estimates and 95% confidence intervals for the prevalence of anti-*T. gondii* antibodies and *T. gondii* DNA were established. These parameters were determined overall, and for each host dependent factors as gender (44 males and 38 females) and age (40 young—less than 5 years old, 21 adults—5–14 years and 21 geriatric—older than 14 years), diagnosed with EIAV or not. The difference in prevalence among groups was statistically analyzed by a test of chi-squared independence. A p value of <0.05 was judged to be statistically significant. All statistics were performed using the EpiInfo 2000 software. Also, the agreement between ELISA and MAT was assessed by calculation of Kappa statistic value using Win Episcope 2.0 program. Kappa value was interpreted as [Petrie and Watson \(1999\)](#): $k \leq 0.20$ without consistency, $0.21 \leq k \leq 0.40$ poor agreement, $0.41 \leq k \leq 0.60$ moderate agreement, $0.61 \leq k \leq 0.80$ good agreement, $k > 0.80$ very good agreement.

4. Results

The prevalence data are presented in [Tables 1 and 2](#). The overall seroprevalence of *T. gondii* infection in horses from Romania was 39% (32/82; 95% CI 28.4–50.4) by ELISA and 37.8% (31/82; 95% CI 27.3–49.2) by MAT. A good agreement (0.768) was obtained between ELISA and MAT. There was no significant difference in the rate of infection between gender and age of animals. Nevertheless, the seroprevalence increased by age: 28.6% in young horses;

Table 2

Prevalence of anti-*Toxoplasma gondii* antibodies by MAT in horses from Romania.

	No. animals	Frequency	Seroprevalence%	CI 95%
Males	44	17	38.64	24.36–54.5
Females	38	14	36.84	21.81–54.01
Young	21	5	23.81	8.22–47.17
Adult	40	17	42.50	27.04–59.11
Geriatric	21	9	42.86	21.82–65.98
EIAV positive	42	23	54.76	38.67–70.15
EIAV negative	40	8	20	9.05–35.65

45% in adults and 38.1% in geriatric horses. Conversely, we noticed that the seroprevalence is significantly higher in EIAV infected horses ($p < 0.002$). No *T. gondii* DNA was found in any heart sample collected from slaughtered horses.

Ten out of 32 positive horses' sera had intensely positive results ($S/P \geq 200$) as measured by the ELISA test. Each of those animals was bioassayed in mice and viable *T. gondii* was isolated from 2 of the seropositive horses. These two horse samples produced brain infections in mice, which remained asymptomatic.

5. Discussion

The estimated prevalence of *T. gondii* in horses can vary with the serological test used. In the present survey, the serum samples were tested by a commercial ELISA and by an in-house MAT. Evaluation of the diagnostic efficiency of ELISA in comparison to MAT revealed that ELISA yielded marginally higher diagnostic efficiency (39%) compared to MAT (37.8%). The present study showed that there were no significant differences between age and gender of animals. Conversely, Boughattas et al. (2011) reported that older horses (>10-year-old) were more likely to be seropositive than horses under 10-years-old, which provided further evidence for the increased risk of *T. gondii* infection with acquisition of age through longer contact with infective oocysts from the environment. The same authors also suggest that male horses are more sensitive to the infection by the parasite than female horses. Significant differences in *T. gondii* seroprevalence were observed in EIAV infected horses. Interestingly, horses infected with EIAV, mostly in the asymptomatic stage of disease, seem more likely to be infected with *T. gondii*. Further investigations warranting the possible interrelation of these two pathogens in horses would be interesting; it would be interesting to know whether infection by one agent predisposes to infection with the other, or whether horses exposed to one agent are also at higher risk of exposure to the other. By PCR no *T. gondii* DNA was found in any heart sample collected from slaughtered horses. This may be explained by the non-homogeneous distribution of *T. gondii* tissue cysts, and the small size of the sample (40 mg) (Juránková et al., 2013). To increase the possibility to isolate *T. gondii* from horse heart samples, we bioassayed only the strongly seropositive samples ($S/P \geq 200\%$) by ELISA, and obtained parasites in two of these ten attempts.

The importance of our study was highlighted by Pomares et al. (2011) who described three cases of toxoplasmosis in humans, caused by atypical strains probably acquired by from ingestion of raw horse meat imported from Canada and Brazil. Considering that in the present study as well as in others (Al-Khalidi and Dubey, 1979; Shaapan and Ghazy, 2007; Evers et al., 2013) viable *T. gondii*

was isolated from slaughtered horses and human toxoplasmosis cases were reported in France in recent years (Elbez-Rubinstein et al., 2009; Pomares et al., 2011), consumption of raw or undercooked horsemeat can be a potential source of human infection. In conclusion, this is the first survey concerning the prevalence of infection with *T. gondii* in horses and also isolating viable *T. gondii* from horses, in Romania. It is important to consider the public health aspects of horse's toxoplasmosis, especially because their meat can be offered for human consumption.

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