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Diagnosis and isolation of *Toxoplasma gondii* in horses from Brazilian slaughterhouses

Diagnóstico e isolamento de *Toxoplasma gondii* em equídeos de frigoríficos brasileiros

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Abstract

This study aimed to investigate anti-*Toxoplasma gondii* antibodies and to isolate the parasite from the brains of horses processed at slaughterhouses in Brazil. We collected brain and blood samples from 398 horses of various ages, from six Brazilian states. Serum samples were evaluated by indirect fluorescent antibody test (IFAT cut-off titre $\geq 1:64$), and brains were submitted to mouse bioassay. Among the 398 horses, positivity for *T. gondii* was identified in 46 (11.6%) by IFAT and in 14 (3.5%) by mouse bioassay. In 12 of those 14 bioassays, mice were positive only by IFAT (cut-off titre $\geq 1:16$), *T. gondii* being isolated in the remaining two. Using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of 18S rDNA to differentiate among *T. gondii*, *Neospora caninum*, and *Sarcocystis neurona*, we found that two of the 14 brains were positive for *T. gondii* only. For genotyping of the two isolates and the PCR-positive brain, we performed PCR-RFLP based on 13 markers, and SAG2 all samples were *Toxoplasma gondii* type I. Collectively, IFAT of horse sera and mouse bioassay identified positivity in 60 (15%) of the samples. Our results show that some horses sent to slaughter in Brazil have been exposed to *T. gondii*.

Keywords: Slaughterhouse, horses, *Toxoplasma gondii*, bioassay, indirect fluorescent antibody test, nested PCR-RFLP.

Resumo

O objetivo do estudo foi investigar anticorpos anti-*Toxoplasma gondii* e isolar o parasita do cérebro de equídeos abatidos em matadouros-frigoríficos no Brasil. Colheram-se amostras de 398 cérebros e sangue de equídeos machos e fêmeas de idades variadas, provenientes de seis estados brasileiros. As amostras de soro foram avaliadas pelo teste de imunofluorescência indireta (IFI) para *T. gondii* (ponto de corte ≥ 64), e os fragmentos de cérebros foram submetidos ao bioensaio em camundongos. Por meio da IFI, 46 (11,6%) equídeos foram soropositivos. Pelo bioensaio em camundongos, 14 (3,5%) cérebros de equídeos testados foram positivos. Em doze dos bioensaios, os camundongos foram positivos somente pela IFI (ponto de corte ≥ 16) e *T. gondii* foi isolado nos outros dois bioensaios. A PCR-RFLP com base em 18S rDNA para diferenciar entre *T. gondii*, *Neospora caninum*, e *Sarcocystis neurona* foram feitas em todos os 14 cérebros e dois foram positivos apenas para *T. gondii*. De dois isolados positivos para *T. gondii* e do cérebro positivo à PCR em que realizou-se a PCR-RFLP, com base em 13 marcadores e SAG2, a genotipagem mostrou ser o *T. gondii* tipo I para todas as amostras. A IFI de soros de equídeos e do bioensaio em camundongos identificaram positividade em 60 (15%) amostras testadas. Os resultados mostram que alguns cavalos enviados para abate foram expostos ao *T. gondii*.

Palavras-chave: Abate, equídeos, *Toxoplasma gondii*, bioensaio, imunofluorescência indireta, nested PCR-RFLP.

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Introduction

In Brazil, horses are bred for sport and recreation rather than for slaughter. Prejudice has traditionally been the principal factor limiting the human consumption of horse meat. This is because, as in many other countries, horses are viewed as pets. However, Brazil has legal provisions allowing the slaughter of horses, as well as the packaging and distribution of horse meat, including a requirement that products containing horse meat be so labelled (JUNQUEIRA et al., 2005).

According to data from the Federal Food Safety and Inspection Service of the Brazilian National Ministry of Agriculture, Animal Husbandry, and Supply (BRASIL, 2012), horse meat is produced by five slaughterhouses, all of which are licensed to export that meat: one in the state of Rio Grande do Sul; two in the state of Paraná; one in the state of Minas Gerais; and one in the state of Bahia. Horse meat is obtained from horses that can no longer work or breed and is produced exclusively for export, because the Brazilian market for horse meat is virtually nonexistent (TORRES; JARDIM, 1987; JUNQUEIRA et al., 2005).

According to Inacio (2010), Brazil ranks fourth among the world's horse meat exporters, accounting for 10.9% of all such export, after Argentina (28.7%), Canada (24.6%), and Poland (14.6%). In 2005, the state of Paraná accounted for 47% of all horse meat exports from Brazil, followed by the states of Rio Grande do Sul, Minas Gerais, and São Paulo (MEDEIROS, 2009).

Contrary to what occurs in Brazil, the use of horse meat for human consumption is a common practice in European and Asian countries, creating a global market and raising concern regarding the consumption of raw or undercooked meat from infected horses, which can be a major route of transmission of *Toxoplasma gondii*.

A recently reported case of congenital toxoplasmosis in a newborn in France where a pregnant woman who was seropositive for *T. gondii* (due to immunization against toxoplasmosis prior to pregnancy) was reinfected, probably after having consumed raw horse meat imported from South America (ELBEZ-RUBINSTEIN et al., 2009) underscores the importance of evaluating the epidemiology of *T. gondii* in exported horse meat.

The prevalence of toxoplasmosis in horses varies by region, diagnosis, and cut-off point. Previous studies have indicated that seropositivity for *T. gondii* in horses ranges from 1.5% to 33% (LARANGEIRA et al., 1985; VIDOTTO et al., 1997; GARCIA et al., 1999; MENDONÇA et al., 2001; NAVES et al., 2005; CAMOSSO et al., 2010).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is used worldwide to determine the genotype diversity of *T. gondii*, as it was in studies conducted by Pena et al. (2008), Dubey and Su (2009), and Yai et al. (2009). Those authors concluded that there is a high diversity in the strains found in Brazil, and none of them observed the type II genotype. However, Macedo et al. (2012) and Da Silva et al. (2011) recently described the type II genotype in cattle and sheep in Brazil, respectively.

The objectives of the present study were to investigate the prevalence of anti-*T. gondii* antibodies in horses processed at slaughterhouses in Brazil and to isolate the parasite from the brains of those horses.

Materials and Methods

1. Collection site and sampling

We collected samples from horses processed at two slaughterhouses, the horse meat batches having been randomly chosen by the Paraná State Branch of the Federal Food Safety and Inspection Service. The target population consisted of approximately 4000 horses. The minimum sample size, as calculated with Epi Info, version 6.04 (DEAN et al., 1994), on the basis of an estimated prevalence of 50%, an error of 5%, and a significance level of 5%, was found to be 384 individuals. We defined the prevalence of 50% to calculate the sample size to determine the prevalence of antibodies against various diseases. The protocols employed in the present study were approved by the Animal Experimentation Ethics Committee of the (Paraná) State University at Londrina, located in the city of Londrina, Brazil (Protocol nos. 59/09 and 60/09).

2. Blood and brain collection

Between July of 2009 and January of 2010, adult horses of various ages were selected from among those arriving from the following states and cities in Brazil:

- State of Paraná (N = 152) – cities of Apucarana (n = 4), Araruna (n = 4), Borrazópolis (n = 4), Cafezal do Sul (n = 15), Campina Grande do Sul (n = 27), Imbituva (n = 13), Londrina (n = 3), Lobato (n = 33), Marquinho (n = 12), Novo Itacolomi (n = 7), Prudentópolis (n = 12), Ribeirão Claro (n = 9), Rio Bom (n = 2), São João do Ivaí (n = 3), and Santa Fé (n = 4);
- State of Minas Gerais (N = 53) – cities of Frutal (n = 33) and Itapagipe (n = 20);
- State of Rio de Janeiro (N=39) – city of Itaipava;
- State of Goiás (N = 6) – city of Caiapônia;
- State of Mato Grosso do Sul (N = 99) – cities of Coronel Sapucaia (n = 10), Deodápolis (n = 21), Paranaíba (n = 22), and Rio Verde de Mato Grosso (n = 46); and
- State of Mato Grosso (N = 49) – cities of Cáceres (n = 20), Canarana (n = 12), and Cuiabá (n = 17).

The horses were selected through systematic sampling, random selection having been carried out daily, depending on the demand at the slaughterhouses.

After the horses had been stunned and exsanguinated, we collected 10-mL blood samples, from each of 398 horses. After clot retraction, the serum was placed in 1.5-mL polyethylene tubes and stored at -15 °C for subsequent serology. Approximately 50 g of brain tissue were collected from each horse. The brain samples were individually stored in isothermal boxes and sent to the State University at Londrina to be analyzed in the Laboratory of Zoonoses and Public Health and in the Protozoology Laboratory.

3. Indirect Fluorescent Antibody Test

The presence of anti-*T. gondii* antibodies in serum was detected by Indirect Fluorescent Antibody Test (IFAT), in accordance with the method proposed by Camargo (1974). We used RH

tachyzoites as antigen, as well as anti-IgG conjugates (Sigma Chemical, St. Louis, MO, USA), together with positive and negative controls for horse and mouse sera. Positivity was defined as a titre $\geq 1:16$ for mice (TSUTSUI et al., 2007) and $\geq 1:64$ for horses (GARCIA et al., 1999).

4. Mouse bioassay for the detection of *T. gondii*

Brain samples from horses were macerated prior to their use in mouse bioassays for the detection of *T. gondii*. All mice used for bioassays were Swiss-Webster albino females weighing approximately 20-25 g. Each processed brain sample was used in order to inoculate two mice intraperitoneally with 1 mL of the sample. Over a six-week period, the mice were observed for clinical signs of infection (ruffled fur or prostration). When one or more clinical signs were observed, the mouse was killed and peritoneal fluid was collected. Fluid samples were mounted on slides, coverslipped, and screened for tachyzoites under light microscopy. After six weeks, we killed all of the surviving mice, after which we collected blood samples for IFAT and brain tissue samples for detection of tissue cyst formation under light microscopy (NAVARRO et al., 1992; DUBEY et al., 1995).

5. PCR-RFLP

The samples that were found to be positive for *T. gondii* by mouse bioassay were submitted to DNA extraction with the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and PCR-RFLP analysis of 18S rDNA (SU et al., 2010), which amplifies a 291-bp fragment in order to differentiate among *T. gondii*, *Neospora caninum*, and *Sarcocystis neurona*. Genotyping was performed by multi-locus PCR-RFLP analysis of 13 markers (SU et al., 2006; PENA et al., 2008): SAG2-3' and SAG2-5' (HOWE et al., 1997); SAG3, GRA6, and SAG1; BTUB; L358; c22-8; c29-2; PK1; Apico; SAG2-alt; and CS3. The positive controls for the samples representing genotypes I, II, and III, as well as for those representing the atypical genotypes, were GT1, PGT, CTG, TgCgCa1, MAS, TgCatBr5, TgCatBr64, and TgRsCr1.

6. Statistical analysis

The statistical analysis was performed with the Epi Info program, version 6.04 (DEAN et al., 1994). Prevalence rates were calculated, and the associations among the results obtained were determined by the chi-square test. The level of significance was set at 5%.

Results

Considering the IFAT and mouse bioassay results together, we detected *T. gondii* positivity in 60 of the 398 samples (15.0%; 95% confidence interval: 11.7-18.9). Forty-six horses (11.6%) were found to be positive for *T. gondii* by IFAT (Table 1), and 14 (3.5%) were found to be positive for *T. gondii* by mouse bioassay. Twelve (3.0%) of the mouse bioassays were found to be positive

by IFAT, and we isolated the parasite in two bioassays because we found tachyzoites in the mouse peritoneal fluid or a cyst in a mouse brain (Table 2).

For the 14 horses that were found to be positive for *T. gondii* by mouse bioassay, we performed PCR on 20 biological samples (14 horse brain samples, three mouse peritoneal fluid samples, two mouse brain samples, and one mouse lung sample). As can be seen in Table 3, we found that three (14.3%) of those samples tested positive: two horse brain samples and one mouse peritoneal fluid sample.

We found significant differences ($p = 0.0001$) among the horses tested in terms of the prevalence of *T. gondii* positivity by region of origin, the lowest prevalence having been found among horses arriving from the central-west region of Brazil (Table 3). The use of PCR-RFLP with the 18S rDNA marker revealed positivity for *T. gondii* alone in 3 horses (on the basis of samples 16, 361, and 404). The *T. gondii* isolates were not amplified in all of the markers used for genotyping, which was probably due to low DNA concentrations (Table 4).

Discussion

In the present study, 11.6% of the horses evaluated were found to test positive for *T. gondii*. This is in agreement with the findings

Table 1. Indirect fluorescent antibody test for *Toxoplasma gondii* in 398 serum samples from horses by origin (region and state), processed in slaughterhouses in the state of Paraná, Brazil, 2009-2010.

Origin			IFAT*
Region	State	City	POS/EXAM ¹ (n/N)
South	PR	Araruna	1/4
	PR	Borrazópolis	1/4
	PR	Cafezal do Sul	1/15
	PR	Campina Grande do Sul	3/27
	PR	Imbituva	5/13
	PR	Lobato	4/33
	PR	Londrina	2/3
	PR	Marquinho	4/12
	PR	Novo Itacolomi	1/7
	PR	Prudentópolis	1/12
	PR	Ribeirão Claro	2/9
	PR	Rio Bom	2/2
Southeast	MG	Itapagipe	2/20
	MG	Frutal	5/33
	RJ	Italva	4/39
Central-west	MS	Coronel Sapucaia	2/10
	MS	Deodópolis	1/21
	MT	Cáceres	1/20
	MT	Cuiabá	4/17
Total			46/398 (11.6%)

*IFAT = Indirect Fluorescent Antibody Test; POS/EXAM = positive/examined; PR = Paraná; MG = Minas Gerais; RJ = Rio de Janeiro; MT = Mato Grosso; MS = Mato Grosso do Sul; ¹Number of sérum samples positive/examined for *Toxoplasma gondii* by IFAT; (cut-off titre $\geq 1:64$).

of Vidotto et al. (1997), who studied 561 serum samples from horses arriving (also for slaughter at one of the licensed slaughterhouses) from the states of Paraná, São Paulo, Mato Grosso, and Mato Grosso do Sul. Using IFAT, those authors found that 14.3% were positive for *T. gondii*. Even 15 years later, our findings are similar to theirs. Garcia et al. (1999) analyzed 173 serum samples from horses on farms in the city of Jaguapitã, Brazil, and found that the prevalence of *T. gondii* positivity was 12.1%.

Table 2. Positivity for *Toxoplasma gondii*, as determined by mouse bioassay, in brain samples from 398 horses slaughtered in slaughterhouses in the state of Paraná, Brazil, 2009-2010.

Origin		Horses		Mouse
Sample/city	IFAT TITRE*	Brain PCR		Bioassay
16/Prudentópolis	1:16	P		P ¹
17/Prudentópolis	≤1:16	N		P ¹
20/Prudentópolis	≤1:16	N		P ¹
25/Campina Grande do Sul	1:16	N		P ¹
237/Lobato	≤1:16	N		P ¹
250/Novo Itacolomi	1:64	N		P ¹
264/Campina Grande do Sul	1:16	N		P ¹
361/Marquinho	≤1:16	N		P ²
364/Marquinho	≤1:16	N		P ¹
404/Lobato	≤1:16	P		P ³
169/Paranaíba	≤1:16	N		P ¹
173/Paranaíba	≤1:16	N		P ¹
177/Paranaíba	≤1:16	N		P ¹
299/Italva	≤1:16	N		P ¹

IFAT = Indirect fluorescence antibody test; PCR = polymerase chain reaction; P = Positive, N = Negative; *cut-off titre ≥1:64; 1. IFAT cut-off titre ≥1:16; 2. Isolation of tachyzoites in peritoneal fluid of mouse; 3. Isolation of brain cysts in mouse.

Table 3. Positivity for *Toxoplasma gondii*, as determined by indirect fluorescent antibody test and mouse bioassay, in horses processed in slaughterhouses in the state of Paraná, Brazil, 2009-2010, by origin (region and state).

Origin		<i>T. gondii</i> *
Region	State	Positive/total n/N (%)
South	Paraná	37/152 (24.2)
Southeast	Minas Gerais and Rio de Janeiro	12/92 (13.0)
Central-west	Goiás, Mato Grosso do Sul, and Mato Grosso	11/154 (7.1)
	Total	60/398 (15.0)

*p = 0.0001.

Table 4. Multi-locus genotyping (polymerase chain reaction- restriction fragment length polymorphism) of *Toxoplasma gondii* strains isolated from horses processed in slaughterhouses.

ISOLATE	CS3	SAG1	5-3SAG2	alt.SAG2	SAG3	BTUB	GRA6	c22-8	C29-2	L358	PK1	APICO
16 ¹	NA	I	I	I	III	I	NA	NA	NA	NA	NA	NA
361 ²	NA	I	I	II	III	II	NA	I	NA	NA	I	NA
404 ³	NA	II/III	I	I	III	I	I	NA	NA	I	NA	NA

NA = not amplified; ¹DNA extracted from horse brain; ²Tachyzoites in mouse peritoneal fluid; ³Bradyzoites in mouse brain.

Mendonça et al. (2001) used IFAT in order to test 343 samples from horses from two regions of Bahia, Brazil, and found that 1.5% were positive for *T. gondii*. Naves et al. (2005) analyzed 117 serum samples from Mangalarga horses on three stud farms in Uberlândia, Brazil, and found the prevalence of *T. gondii* positivity to be 5.1%. Camossi et al. (2010) tested 253 serum samples from horses in Botucatu, Brazil, and found the prevalence of *T. gondii* positivity to be 0.4%. However, Larangeira et al. (1985) used IFAT to evaluate 750 horses on farms in the state of Mato Grosso do Sul and found 27.6% prevalence of *T. gondii* positivity.

The above mentioned differences might be due to various factors: equine resistance to infection; environmental conditions; the strain of *T. gondii*; the type of feed given and the source of the water provided; and the population density of domestic or wild cats in the environment (KIJLSTRA; JONGERT, 2009; KOUAM et al., 2010; MENDONÇA et al., 2001; NAVES et al., 2005; TENTER et al., 2000; VIDOTTO et al., 1997). According to Kouam et al. (2010), the type of activity and the geographic location of the horses have a significant effect on the development of *T. gondii* infection.

In the present study, horses that were found to be positive for *T. gondii* by mouse bioassay were not found to be positive by IFAT. In a study conducted in Australia, in which the authors used the latex agglutination test in order to test serum samples from a female dog and her six puppies for *T. gondii*. The dog tested negative, and the results of all of the tests performed subsequently (including those of those performed up to six months after the birth of the six puppies) were also negative. However, Western blotting and PCR showed evidence that the dog had been infected with *T. gondii*. Although the latex agglutination test revealed that the six puppies were negative for *T. gondii*, PCR showed that they had *T. gondii* DNA in their brain tissue (AL-QASSAB et al., 2009).

Araújo et al. (2010) studied 43 rodents in the urban area of Umuarama, Brazil, using blood samples for the modified agglutination test, as well as brain and heart samples for bioassay. Although all of the rodents tested negative for anti-*T. gondii* antibodies, the parasite was isolated in one mouse (*Mus musculus*) and one rat (*Rattus rattus*).

According to Dubey et al. (1999), the risk of becoming infected with *T. gondii* by consuming horse meat is low because the prevalence of *T. gondii* in horses is low. However, the previously mentioned case of congenital toxoplasmosis in an infant born to a pregnant woman who probably ate raw horse meat in France (ELBEZ-RUBINSTEIN et al., 2009), together with the fact that there have been few studies employing bioassay to screen for toxoplasmosis in horses, reignites this discussion. In the present

study, we found brain cysts and tachyzoites in mice inoculated with macerated horse brain. This provides evidence that tissue cysts in horses can transmit *T. gondii*.

Although the Brazilian National Ministry of Agriculture, Animal Husbandry, and Supply Food Safety and Inspection Service complies with the international standards regulating horse meat, *T. gondii* cysts are invisible to the naked eye. In order to prevent *T. gondii* infection, the meat should be stored frozen and subsequently cooked appropriately. The cysts found in contaminated meat can be destroyed by storing the meat at -20°C for 18-24 hours (REMINGTON et al., 1995).

If we take into consideration the fact that horse meat production involves the slaughter of horses raised in a variety of ways and under varying sanitary conditions, quality management in horse breeding can aid in reducing the risk of infection with etiologic agents such as *T. gondii*, which can be transmitted by eating meat or viscera, in regions where horse meat is habitually consumed. On July 1, 2010, the slaughterhouses licensed by the Brazilian National Ministry of Agriculture, Animal Husbandry, and Supply had to make adjustments in order to comply with new guidelines, which include the requirement for horses to have remained on the same farm for six months before slaughter, as well as for the owners of those farms to maintain a list of the veterinary drugs used during that period. From a food safety standpoint, the fact that Brazil devised a plan in order to meet the new requirements and, more importantly, the fact that the plan was approved by the Food and Veterinary Office of the European Union indicate that a great effort was made in order to avoid sanctions (MOREIRA, 2010).

Although it is not consumed by Brazilians, horse meat from Brazil constitutes a route of transmission of *T. gondii* to humans in other locations. Some of the horses investigated in the present study were found to have been infected with *T. gondii*. Mouse bioassay and PCR demonstrated the presence of *T. gondii* in horse brain, which suggests the presence of cysts in other organs, as well as in muscles. Our results show that some horses sent to slaughter in Brazil have been exposed to *T. gondii*.

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