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Toxoplasma gondii in milk of naturally infected dairy ewes on west mesoregion of Santa Catarina state, Brazil

[Toxoplasma gondii *em leite de ovelhas leiteiras naturalmente infectadas na mesorregião oeste de Santa Catarina, Brasil*]

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ABSTRACT

This study aimed to detect *Toxoplasma gondii* in the milk of dairy sheep in the Western mesorregion of state of Santa Catarina by bioassay (22 milk samples from eight ewes seropositive; IFA ≥256) and PCR [for the detection of agent in the brains of mice inoculated on bioassay and directly from milk (108 samples from 42 seropositive ewes (IFA, ≥64) in different lactation periods)]. *T. gondii* DNA was detected in mice brains inoculated with milk from eight sheep (a sample of the 45th day of lactation and seven in the collection of 90th day) and directly from the milk in samples of the second collection (90 days) in five animals. Taking into account both assays, from a total of 42 ewes in lactation and seropositive for *T. gondii*, 30.95% (13/42) of the animals presented evidences of *T. gondii* presence in milk. Positive PCR samples were sequenced and the results confirmed ≥97% identity with the membrane antigen P22 gene of *T. gondii*. The results showed that *T. gondii* is present in the milk of sheep, representing a possible source of infection to humans through the consumption of milk "in natura" and/or derivatives, besides the possibility of lactogenic transmission to lambs.

Keywords: *Toxoplasma gondii*, milk sheep, PCR, Santa Catarina state

RESUMO

O objetivo deste estudo foi detectar Toxoplasma gondii *no leite de ovinos leiteiros na mesorregião oeste de Santa Catarina, por meio do bioensaio (22 amostras de leite de oito ovelhas soropositivas para* T. gondii *- RIFI ≥256) e PCR [nos cérebros de camundongos inoculados no bioensaio e diretamente do leite (108 amostras de 42 ovelhas soropositivas (RIFI ≥64) em diferentes períodos de lactação)]. DNA de* T. gondii *foi detectado no cérebro de camundongos inoculados com leite das oito ovelhas (uma amostra do dia 45 e sete do dia 90 de lactação) e diretamente do leite em amostras da segunda coleta (90 dias de lactação), em cinco animais. Considerando os resultados de ambos os ensaios, de 42 ovelhas em lactação e soropositivas para* T. gondii, *30,95% (13/42) dos animais apresentaram evidências da presença do parasito no leite. As amostras positivas na PCR foram sequenciadas e os resultados confirmaram ≥97% de identidade com o antígeno de membrana gene P22 de* T. gondii. *Os resultados mostraram que o* T. gondii *está presente no leite de ovelhas, o que representa uma possível fonte de infecção para os seres humanos, por meio do consumo de leite* in natura *e/ou de derivados, além da possibilidade de transmissão lactogênica aos cordeiros.*

Palavras-chave: Toxoplasma gondii, *leite ovino, PCR, Santa Catarina*

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INTRODUCTION

Toxoplasma gondii is a protozoan that can infect warm-blooded animals including humans. It is highly prevalent in many areas of the world, and important in human medicine and veterinary (Tenter *et al*., 2000).

In Brazil, studies have reported wide distribution of sheep toxoplasmosis and occurrence of anti-*T. gondii* antibodies ranging between 7.7% and 52% (Silva and Langoni, 2001; Lopes *et al*. 2010).

Most cases of human toxoplasmosis are attributed to the consumption of raw meat or food and water infected with oocysts. However, Skinner *et al*. (1990) emphasize that the consumption of contaminated milk can cause serious clinical disorders in immunocompromised children and pregnant women and Tenter *et al*. (2000) reported seroconversion to *T. gondii* in children taking the goat milk regularly.

The presence of *T. gondii* tachyzoites has been reported in the milk of sheep, goats, cattle and mice (Chiari and Neves, 1984; Vitor *et al*., 1991; Remington *et al*., 2004, Ragozo *et al.*, 2009; Camossi *et al*., 2011).

Since clinical toxoplasmosis in humans associated with unpasteurized goat milk consumption has already been registered (Chiari and Neves, 1984), the biological similarities between species to suggest that sheep milk is also considered a potential source for human infection, making it important to research *T. gondii* in this fluid.

MATERIAL AND METHODS

For the study, 108 samples of milk (42, 37 and 29 samples at 45, 90 and 120 days of lactation, respectively) were collected from 42 lactating ewes that were seropositive (IFA) to *T. gondii*, with antibody titers of 1:64 (26) 1: 256 (12) 1 1024 (three) and 1: 4096 (one) to research the agent by PCR and bioassay. The samples were from animals of a farm of dairy sheep, located in Chapecó, West messoregrion of the state of Santa Catarina. In a previous study (unpublished data), sheep in this farm showed occurrence of antibodies against *T. gondii* of 12.4% (39/298). The sheep were bred in semi-extensive system, with native pasture diet and supplemented with feed. The average age of lactating ewes was 1.5 years.

IFA for IgG anti-*T. gondii* was performed using RH strain tachyzoites as antigens. Negative and positive control serums were added to each slide and anti-sheep or anti-mice antibody conjugated to fluorescein isothiocyanate that were used. The IFA cut-off value was 1:64 (for sheep and mice) and the positive samples were subjected to four-fold dilutions, until the maximum titration reaction was reached.

The samples (10mL each) were obtained by manually milking the teats and were collected in sterile polypropylene tubes DNAses and RNAses free and kept under refrigeration (4-8°C) until arrival at the Laboratório de Parasitologia e Doenças Parasitárias do Centro de Ciências Agroveterinárias (CAV) da Universidade do Estado de Santa Catarina (UDESC) in Lages, SC. Two aliquots (3mL) of milk were extracted and stored at -20Cº for PCR. The remaining sample was used for the bioassay.

For PCR, 4µL of extracted DNA (quantified using the Nanodrop® kit according to the manufacturer's instructions) from milk samples (250µL) was added to 21µL of a mixture of 3.0 mM of each primer, 1.0mM dNTPs, 1.0mM MgCl2 and 0,2U of Taq DNA polymerase. Amplification of parasite DNA was performed in a thermocycler: Five minutes at 94°C for denaturation in a single cycle, followed by 35 cycles of one minute at 94ºC for denaturation, one minute at 60°C for annealing and one minute at 72°C for extension, followed by a final extension of seven minutes at 72°C. The PCR products were subjected to agarose gel electrophoresis 2% stained with "red gel". DNA of the *T. gondii* VEG strain were used as positive control, and ultrapure water as negative control (Moura *et al*., 2011). The SAG2R4 (59GCATCAACAGTCTTCGTTGC39) and SAG2F4 (59GCTACCTCGAACAGGAACAC39) primers were used to amplify a DNA segment of 332 Kd.

Of the 108 samples of milk obtained, 23 from eight lactating ewes (Table 1) were selected because they had higher titers of antibody [1: 256

(six), 1: 1024 (one) and 1: 4096 (one)] and were used in the bioassay.

For bioassay Swiss albino mice were used, of both sexes over the age of two months from the vivarium of the Universidade do Estado de Santa Catarina (UDESC) (CAV/UDESC). Milk samples (7mL) were centrifuged at 2.500rpm for 10 minutes. The supernatant was discarded and to the sediment antibiotic was added [Benzylpenicillin benzathine 3.000,000UI, Benzylpenicillin procaine 1.500.000UI, Benzylpenicillin Potassium 1500.000UI, Dihydrostreptomycin base (sulfate) 1.250mg and Streptomycin base (sulfate) 1.250mg] at a dose of 0.5mL/2mL sample. Following brief manual homogenization, each sample was divided into two equal portions (approximately 1.0 mL) and inoculated intraperitoneally in two animals, which were identified and maintained in suitable boxes, with water and food "ad libitum". The inoculated mice were observed daily for the verification of clinical signs of acute infection. After eight weeks, the animals were euthanized (according to principles of animal welfare) for blood and brain collection for serology (IFA) and search for the parasite (by Squash technique and PCR), respectively.

From PCR positive samples (milk and brain), the DNA was purified and ligated into pGem Easy Vector® following the manufacturer's specifications. Briefly, 1µL were added pGEM, 1µL of T4 DNA ligase, 7µL of purified PCR product and 1µL of ultrapure water. These were ligated for 16 hours at 16°C and subsequently transformed into competent DH10B calcium. The colonies were screened directly by PCR and the positives were grown in LB liquid medium and then the plasmid DNA was extracted for sequencing

The sequencing of the samples was performed in High Performance Technology Center Laboratory in Life Sciences (LacTad) of the State University of Campinas (UNICAMP), Campinas / SP, using the automatic sequencer ABI 3730XL (Genetic Analyze). The mold 250ng DNA was labeled using T7 promoter primer 2,5pmol AAT ACG ACT CAC TAT AGG and 3mL BigDye Terminator v3.1 reagent (Applied Biosystems) in a final volume of 10mL. The labeling reactions were performed in a thermocycler with an initial denaturation step at 96°C for 3min, followed by 25 cycles of 96°C for 10sec, 55°C for 5sec and 60°C for 4min. The precipitated product was diluted in 10mL formamide, denatured at 95°C for 5min, cooled on ice for 5min and eletroinjetados in automated sequencer. The sequences were analyzed by NucleotidBlast® tool available at http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, using the parameters "Highly similar sequences (megablast).

This research project was approved by the Ethics Committee for Animal Experimentation of the CAV/UDESC under protocol – 1.03.12

RESULTS AND DISCUSSION

Considering the results of both techniques, for 42 lactating ewes and positive for *T. gondii*, the parasite DNA was detected in milk of 30.95% (13/42) of the animals and in 12.04% (13/108) of the samples. In no sheep *T. gondii* was detected in more than one milk collection (Table 1).

Directly from milk, the agent was detected in 4.63% (5/108) of the samples, all from the second collection (90 days of lactation) in five animals. In the samples of the first and third milk collection no *T. gondii* DNA was detected.

By bioassay, *T. gondii* DNA was detected in the brains of mice inoculated with milk all eight sheep (one in the first sampling, day 45, and seven in the second collection, day 90) (Table 1).

The PCR was used to search the agent in milk, because it is a good indicator of parasite presence in the analyzed material (Mancianti *et al*., 2013). Although the presence of DNA of the parasite does not indicate the viability of the agent nor its potential infectivity, Moura *et al*. (2007) observed that PCR have advantages over traditional methods (bioassay) and research shows a good correlation between PCR and bioassay (Homan *et al*., 2000; Dehkordi *et al*, 2013.).

All samples have from 97-100% identity with the membrane antigen P22 gene of *T. gondii* (GenBank: AB667972.1).

Table 1. Detection of *Toxoplasma gondii* (bioassay and PCR) in milk samples from sheep, the West mesorregion of Santa Catarina State, collected at 45, 90 and 120 days of lactation (collections 1, 2 and 3)

Ewe	IFA	BIOASSAY			PCR MILK		
		Collect 1 n/S/PCRc	Collect 2 n/S/PCRc	Collect 3 n/S/PCRc	Collect 1	Collect 2	Collect 3
2	1:256	2/-/ +	2/-/-	2/-/-	-	-	-
3	1:1024	2/-/-	2[1]/-/ +	2/-/-	-	-	-
42	1:256	NR	NR	NR	-	+	-
5	1:64	NR	NR	NR	-	-	-
6	1:64	NR	NR	NR	-	NR	NR
7	1:64	NR	NR	NR	-	-	-
8	1:64	NR	NR	NR	-	-	-
110	1:64	NR	NR	NR	-	+	-
10	1:64	NR	NR	NR	-	NR	NR
11	1:256	2[1]/0/-	2 /0/ +	2/0/-	-	-	-
13	1:256	2/0/-	2/0/ +	2[1]/0/-	-	-	-
14	1:256	NR	NR	NR	-	-	-
17	1:64	NR	NR	NR	-	-	-
19	1:64	NR	NR	NR	-	-	-
39	1:1024	NR	NR	NR	-	+	-
23	1:256	NR	NR	NR	-	-	-
24	1:4096	2/0/-	2[1]/0/ +	NR	-	-	NR
28	1:256	2/0/-	2/0/ +	2/0/-	-	-	-
31	1:256	NR	NR	NR	-	-	-
32	1:64	NR	NR	NR	-	NR	NR
102	1:64	NR	NR	NR	-	-	-
91	1:64	NR	NR	NR	-	-	-
131	1:256	2/0/-	2[1]/0/ +*	2/0/-	-	-	-
61	1:1024	NR	NR	NR	-	-	-
86	1:64	NR	NR	NR	-	+	-
67	1:64	NR	NR	NR	-	-	-
54	1:64	NR	NR	NR	-	-	-
52	1:64	NR	NR	NR	-	NR	NR
79	1:64	NR	NR	NR	-	-	-
99	1:64	NR	NR	NR	-	-	-
45	1:64	NR	NR	NR	-	NR	NR
147	1:64	NR	NR	NR	-	-	-
56	1:256	2/0/-	2/0/ +	2[1]/0/-	-	-	-
102	1:256	NR	NR	NR	-	-	NR
120	1:64	NR	NR	NR	-	NR	NR
136	1:64	NR	NR	NR	-	-	-
150	1:64	NR	NR	NR	-	NR	NR
149	1:64	NR	NR	NR	-	-	-
129	1:64	NR	NR	NR	-	-	-
78	1:64	NR	NR	NR	-	+	-
124	1:64	NR	NR	NR	-	-	-
133	1:64	NR	NR	NR	-	NR	NR

n: Number of mice inoculated in the bioassay
S: +/- = Search result brain cysts through the technique of "Squash"
PCRc: +/- = PCR result of the brains of mice inoculated with milk (* = two mice with positive brain PCR).
[1]: mouse died during the bioassay.
NR = not done

Of the 42 lactating ewes, 30.95% (13/42) showed evidence of the presence of parasite in milk. Higher rate results to those observed by Camossi *et al*. (2011), in São Paulo, e Santana Rocha *et al*. (2015), in Bahia, which identified 25% (5/20) and 6.5% (18/275), respectively, of the sheep excreting *T. gondii* by milk.

In 12.04% (13/108) of samples from milk sheep, in this study, *T. gondii* DNA was detected. Similar results to those obtained by Camossi *et al*. (2011), in São Paulo, and Moura *et al*. (2011), in Bahia, which reported 10% (7/70) and 10.5% (21/200) of milk samples from sheep with the presence of the parasite. Also similar results, but in goats, were reported by Mancianti *et al*. (2013) that detected DNA of *T. gondii* (PCR) in 13% (10/77) of samples of milk from animals naturally infected in Italy. Lower values were reported in Fusco *et al*. (2007), in Italy, and Tavassoli *et al*. (2013), in Iran, they found 3.4% and 4.6%, respectively, of milk samples from sheep with the presence of *T. gondii*.

Camossi *et al*. (2011) analyzed, through PCR, 69 milk samples from seronegative sheep and did not find the agent and, therefore, in this study, *T. gondii* was searched only in milk from seropositive sheep. However, Bezerra *et al*. (2015), in Pernambuco, Brazil, detected *T. gondii* DNA in 15 milk samples from naturally infected goats, of which only five had antibodies (IFT, ≥ 1:64) against the agent.

In none of the inoculated mice was verified the presence of cysts by the methodology of "squash". This result is due probably to the low concentration of tachyzoites in this fluid (milk), insufficient to cause clinical disease in mice and/or induce the formation of cerebral cysts in an amount sufficient to be detected by this technique.

All mice were seronegative for *T. gondii* (IFAT, ≥1: 64). This result may reflect the low parasite load found in milk, the virulence of the strain and the individual animal immune response that can generate antibody levels undetectable in the dilution of 1:64. The prozone phenomenon (Richtzenhain and Soares, 2006) can also influence the results obtained in this study. Still, Rosa *et al.* (2001) and Trevisani (2013) found that mice inoculated, even with cysts on SNC and/or tachyzoites not seroconverted, while others were seropositive (IFA 1:16) without the detection of tissue cysts (Trevisani, 2013). Divergences between results of isolation agent by bioassay and serology of the inoculated mice were also observed by Holsback *et al*. (2012) and Cademartori *et al*. (2014).

The presence of the agent in milk may be the effect of physiological changes in the immune system at the time of peripartum, which favor the conversion of bradyzoites in tachyzoites (process which can be caused by various phenomena of immune order, mainly cellular immunity) and thus could reach the milk (Camossi *et al*., 2011). The papers show that tachyzoite is the evolutionary phase normally present in milk (Dubey and Beattie, 1988) and, although they can be inactivated by the gastric juice, Cook *et al*. (2000) presented results that indicate that the ingestion of raw milk, and/or their derivatives, can cause human infection. This is possible if they penetrate the oral and/or pharyngeal mucosa before reaching the stomach (Sacks *et al*., 1982) or a part of tachyzoites excreted in the milk which is not destroyed by the gastric juice due to its rapid passage through this digestive compartment (Tavassoli *et al*., 2013). Dubey (1998) showed that within two hours tachyzoites resist in solution pepsin and trypsin and cats can acquire the infection by ingestion of large numbers of tachyzoites. Moreover, Walsh *et al*. (1999) observed that tachyzoites remain viable in goat milk up to seven days at 4°C, indicating the risk of consumption of "in natura" milk. Also, in cow's milk, artificially infected with cysts (Hiramoto *et al*., 2001) of *T. gondii*, the agent remained viable and can be a source of infection for humans. One hypothesis, not evaluated, is that the milk fat may offer some protection to *T. gondii* tachyzoite regarding the action of gastric juice.

Human toxoplasmosis through ingestion of raw goat milk (Skinner *et al*., 1990) has already been registered (Chiari and Neves, 1984) and, in state of Minas Gerais, Chiari *et al*. (1987) found a correlation between human infection and the consumption of goat milk. While epidemiologically these accounts have no impact, in terms of human health they are of concern, even when only isolated cases. Furthermore, the similarity between goats and sheep suggest that the same can occur with sheep milk. Still, the contamination of sheep milk with tachyzoites of *T. gondii* should not be

overlooked, as some homemade cheeses produced from this raw material, are usually eaten fresh and may pose a risk to human health if they are produced from unpasteurized milk (Fusco *et al*., 2007). The milk production in the studied property is only intended for human consumption "in natura" and its derivatives.

In this study, most of the animals have the infection in the chronic phase, as shown by the results of ewe's serology. These results demonstrate that sheep, even healthy, may excrete the parasite in milk. In Brazil, Camossi *et al.* (2011) detected DNA of *T. gondii* in 10% from milk (7/70) of naturally infected sheep also in the chronic phase of the disease.

In this study, *T. gondii* DNA was observed in milk samples from 13 animals in only one of the three milk collections over the period (45, 90 and 120 days of lactation) not featuring intermittency. Powell *et al.* (2001) found (PCR) and/or isolated (Bioassay) *T. gondii* in milk samples from five out of six cats experimentally infected with cysts of different strains of the parasite, intermittently. These results, according to the authors, could be related to the volume of samples or the sporadic release of the parasite. Still, the presence of *T. gondii* in cat milk over three weeks of lactation may be responsible for the infection of kittens that are born healthy, congenitally infected, but sicken and die. Despite the differences between species, the same might occur with lambs.

CONCLUSION

Toxoplasma gondii DNA in the milk of naturally infected sheep indicates the presence of the agent, possibly tachyzoites, if feasible, representing a possible source of infection for humans through milk consumption "*in natura*" and/or derivatives, beyond the possibility of lactogenic transmission for lambs.

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