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Eco-Epidemiology of Toxoplasmosis in Ruminant and the Experimental Model Evidence from Mice Bioassay for Transmission of Infection Starting of Contaminated Soil Samples Confirmed by Polymerase Chain Reaction (PCR) and Indirect Immunofluorescence Reaction (RIFI) Techniques Diagnostic

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Abstract: The present work aims had as to determine the occurrence of toxoplasmosis in sheep and cattle herds in the region of Sorocaba-SP, Brazil and the presence of oocysts in soil samples through of bioassay in mice and PCR. Two hundred seventy-two sheep and seventeen cattle coming from four farms in the region of Sorocaba-SP, being two exclusive of sheep and two associated with cattle were studied. Indirect immunofluorescence reaction (RIFI) for detecting anti-*Toxoplasma gondii* antibodies was utilized. The detection of *T. gondii* oocysts in soil samples was performed by bioassay in mice inoculated intraperitoneally (IP) and orally (VO). On days 0, 30 and 60 after inoculation, samples were collected from and analyzed by RIFI for observation of seroconversion. After 60 days of inoculation, the mice were euthanized and organs included mice analyzed by polymerase chain reaction (PCR). Soil samples were analyzed for of detecting *T. gondii* DNA by the PCR. The positivity of the herds was of 31.62% for sheep and 94.12% cattle serum reagents by RIFI. The antibodies titers for both herds ranged from 1:16 to 1:1024. The positivity of mice inoculated IP was 70% (14/20) and of 63.33% (19/30). Seroconversion in mice ranged from 1:16 to 1:256 (IP) and 1:16 to 1:32 (VO) examined by PCR. The positivity of mice organs was of 30% (6/20) to the lungs of mice via IP versus 23:33% (7/30) inoculated VO route, being -16.67% lung, 3.33% liver and 3.33% for brain - mice inoculated via VO. Based on the results and in the evidence from rodent models in experimental conditions of this study, can conclude that soil contaminated by *T. gondii* oocysts in the felids was the main factor predisposing of infection by toxoplasmosis in sheep farms.

Key words: Sheep · Oocysts · *Toxoplasma gondii* · PCR · Soil · RIFI

INTRODUCTION

Toxoplasma gondii, was initially called *Leishmania gondii* and discovered by French parasitologists Nicolle and Manceaux [1] as a parasite in an African rodent (*Ctenodactylus gondii*) which was considered his host or reservoir. In São Paulo of State, Splendore [2] described the presence of parasites in rabbits and Darling [3] observed it in humans.

In 1909, Nicolle and Manceaux [4] explained the meaning of the parasite *T. gondii*. They mentioned that Toxo mean arc and plasma refer to germinal material, namely, the arch-shaped body.

The intracellular apicomplexan protozoan *T. gondii* is found worldwide. The members of the cat family *(Felidae)* are the definitive hosts, while mammals and birds are intermediary hosts. It has an important pathogenic role in fetal medicine.

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The life cycle of the family Sarcocystidae [5] have two types of hosts, the definitive and intermediate (non-obligatory). In the definitive host stages of the parasite entero-epithelial and extra-intestinal (in other epithelial tissues), occur, while in the intermediate host extra-intestinal occurs [6]. The parasite undergoes full gametogenesis and mating within the intestinal epithelium, culminating in the generation of oocysts that are shed in the faeces of cats. The oocysts are highly infectious and extremely stable in the environment. Owing of this model of biological cycle, the *T. gondii* is considered polyxenic and heteroxenic facultative.

T. gondii, has three infectious stages [7] in the definitive hosts: the tachyzoites (grouped), bradyzoites (tissue cysts) and sporozoites (oocysts), while in intermediate hosts are found tachyzoites and bradyzoites.

The tachyzoites, bradyzoites and sporozoites are ultra structurally similar, but they differ in some organelles and inclusion bodies. They are also similar to each other in attacking and penetration of host cells.

The tachyzoites possess a form of semi-arc and measure 4 - 8 μ long by 2-4 μ width [8]. They are found during the acute phase of infection [9]. In response to host immunity, they differentiate into bradyzoites [8].

The tissue cyst has a high affinity for nerve and muscle tissue, predominantly located in the central nervous system, eyes and skeletal muscles of the heart, being more rarely found in visceral organs like lungs, liver and kidneys.

The life cycle of *T. gondii* was elucidated in the small intestine of cats (entero-epithelial cycle) and oocysts in their feces [10].

The cats may shelter oocysts after ingesting any of the three stages of *T. gondii* [7].

Of agreement to the latest Census of Agriculture conducted in 2006, sheep and cattle in Brazil were estimated at 13.9 and 169.9 million, respectively. The Northeast region had 7.7 millions sheep and 26 millions cattle. The North had 474,000 of sheep and 31.2 millions cattle. The Center-west region had 867,000 of sheep and 53.8 million of cattle. The South region was the holder of 3.9 million of sheep and 23.9 million of cattle and Southeast totaled 763,000 head of sheep and 34.9 million of cattle [11].

The toxoplasmosis in the animals, is related to the ingestion of oocysts that present in the food and contaminated soil [12, 13].The ingestion of oocysts is the principal route of transmission for herbivores, mainly sheep and goats [14, 15]. The outbreaks of toxoplasmosis in humans may be related to environmental contamination by oocysts [6], or by a habit of certain ethnic groups, relative to the handling and consumption of meat or offal from sheep, raw or under-cooked [16]. This is justified, since in the animals slaughtered for consumption, the inspection is hampered because it is an infection in which clinical signs and lesions are unapparent. Mainly due to this clinic-pathologic characteristic, the control of this zoonosis is hindered [17].

Isaac-Renton *et al.* [18], showed the possibility of dissemination of non-sporulated oocysts through water surface.

Important studies have been applied to search for *T. gondii* in herds sheep and cattle by Indirect Immunofluorescence Reaction (RIFI), being the frequencies found between these of: sheep: 34.7% (207/597) [19], 23% [20], 54.6% (185/339) [21], 51.8% (188/228) [22], 51.47% (157/305) [23], 35.3% (61/173) [24], 43.2% (307/711) [25] 35.41% (364/1028) [26] and in dairy cattle of: 48.51% [27], 25.8% [22], 41.4% (144/348) [28], 71% (1420/2000) [29], 11.83% (71/600) [30] and 14.77% (87/598) [31].

This study was carried an exclusive sheep farms and sheep farms intercropping with cattle grazing at Sorocaba, Sao Paulo State, Brazil. The objective of this study is to determine the incidence of *Toxoplasma gondii* in sheep and cattle by RIFI and in soil samples by polymerase chain reaction (PCR).

MATERIALS AND METHODS

Two hundred and eighty nine ruminants serum samples were collected from four herds which composed of seventeen cattle and two hundred seventy-two sheep herds, totalizing four herds of sheep and two herds cattle, being these two last herds ovine (144 sheep) intercropping with bovines, all located in the municipality of Sorocaba, Sao Paulo State, Brazil. The animals were farms in conditions extensive grazing. Salt was available in troughs and water in various types of drinker (Tables 1 and 2).

In the eco-epidemiological studies of toxoplasmamosis in these sheep-farms was taken into account the promiscuity of domestic felids with farm animals and finding pasture soil contaminated with cat feces. The samplings were conducted between February-March 2008.

Table 1: Anti-*T. gondii* of antibodies titers by indirect immunofluorescence reaction (RIFI) in serum of mice inoculated bioassay by intraperitoneal (IP) and oral (VO) routes, with samples of soil collected in farms with sheep-farms exclusive and sheep-farms associated with cattle

		IP Titers			VO Titers	
	n.° of mice			n.° of mice		
sheep-farm /site of collect	POS/INOCULATED IP	30 dpi	60 dpi	POS/INOCULATED VO	30 dpi	60 dpi
A/ Sheepfold	2/2	64; 128	32; 128	2/3	NR; 16; 32	2 NR; 16
A/ Interior of the bays	2/2	16; 256	NR; 128	2/3	NR; 16; 32	2 NR; 16
A/ Soil with cat feces	1/2	NR; 64	NR; 128	1/3	2 NR; 16	2 NR; 16
Subtotal	**5/6 (83.33%)**			**5/9 (55.56%)**		
B/ Sheepfold	1/2	NR; 32	NR; 16	3/3	2 NR; 16	16; 16; 16
B/ Interior of the bays	1/2	NR; 32	NR; 16	0/3	3 NR	3 NR
Subtotal	**2/4 (50.00%)**			**3/6 (50.00%)**		
C/ Sheepfold	1/2	NR; 16	2 NR	2/3	NR; 16; 16	2 NR; 16
C/ margin of lake	2/2	16; 64	NR; 64	1/3	2 NR; 16	3 NR
Subtotal	**3/4 (75.00%)**			**3/6 (50.00%)**		
D/ Interior of the bays	1/2	2 NR	NR; 16	2/3	2 NR; 16	2 NR; 16
D/ Sheepfold	2/2	16; 16	NR; 16	3/3	NR; 16; 16	2 NR; 16
D/ Margin of lake	½	2 NR	NR; 16	3/3	2 NR; 16	16; 16; 16
Subtotal; [range titer total]*	4/6 (66.67%)	12/20 [(60%); titers:	10/20 [(50%); titers:	8/9 (88.89%)	13/30 [(43.33%);	12/30 [(40%);
Total	14/20 (70%)	16 – 256]	16 – 128]	19/30 (63.33%)	titers: 16 – 32]	titers: 16]

* The percentage of RIFI titers serum reagents from mice inoculated with soil sampling showed no difference (P>0.05) significant for the totals response between intraperitoneal (IP) and oral (VO) routes, at 30 and 60 days post inoculation (dpi) in the different properties. [1]dpi: days post inoculation; NR: Not reagent.

Table 2: Diagnostic by PCR for the presence of *T. gondii* in organs (tissues) of mice inoculated bioassay by intraperitoneal (IP) and oral (VO) routes with supernatant sampling of soil

		Intraperitoneal route (IP)				Oral route (VO)		
		PCR in the tissues studied N° POS / N° NEG				PCR in the tissues studied N° POS / N° NEG		
Sheep-farm/	Mice				Mice			
site of collect	POS/INOC	Lung*	Liver	Brain	POS/INOC	Lung	Liver	Brain
A/ Sheepfold	1/2	1 P / 1 N	2 N	2 N	1/3	1 P / 2 N	3 N	3 N
A/ Interior of the bays	1/2	1 P / 1 N	2 N	2 N	0/3	3 N	3 N	3 N
A/ Soil with cat feces	2/2	2 P	2 N	2 N	2/3	2 P / 1 N	3 N	3 N
B/ Sheepfold	0/2	2 N	2 N	2 N	0/3	3 N	3 N	3 N
B/ Interior of the bays	0/2	2 N	2 N	2 N	0/3	3 N	3 N	3 N
C/ Sheepfold	1/2	1 P / 1 N	2 N	2 N	1/3	3 N	1 P / 2 N	3 N
C/ Margin of lake	0/2	2 N	2 N	2 N	0/3	3 N	3 N	3 N
D/ Margin of lake	1/2	1 P / 1 N	2 N	2 N	1/3	3 N	3 N	1 P / 2 N
D/ Sheepfold	0/2	2 N	2 N	2 N	0/3	3 N	3 N	3 N
D/ Interior of the bays	0/2	2 N	2 N	2 N	2/3	2 P / 1 N	3 N	3 N
Total (site): 10	6 P / 20	6 P/ 14 N	20 N	20 N	7 P/30	5 P / 25 N	1 P / 29 N	1 P / 29 N
	(30% P)	(30% P)	(100% N)	(100% N)	(23.33% P)	(16.67% P)	(3.33% P)	(3.33% P)

*The percentage of positive (P) by PCR from tissues of the lung, liver and brain of mice inoculated with soil sampling showed no difference (P>0.05) significant for the totals of response between intraperitoneal (IP) and oral (VO) routes. N° mice POS/INOC: number of positive on the total mice inoculated, P: positive, N: negative.

The serum of sheep and bovine were subjected to indirect immunofluorescence reaction (RIFI), for determining the presence of *T. gondii* antibodies according to Camargo [32]. Serums were diluted in buffered saline (PBS), pH 7.4. The proportion of serum to PBS was 1µL to 64 µL have PBS. Were placed on plates previously sensitized with tachyzoites of *T. gondii* RH strain stained, using the following procedure: conjugated sheep anti-IgG, labelled with fluorescein isothiocyanate (affinity Purified Antibody Fluorescein rabbity anti-sheep IgG), diluted 1:250 in Evans Blue solution at 0.001% and conjugated bovine anti-IgG labelled with fluorescein isothiocyanate (affinity Purified Antibody Fluorescein rabbity and cattle anti-IgG) diluted 1:300 in Evans Blue

solution at 0.001%. The blade assembly-cover slip was performed using buffered glycerin pH 8. All these steps were carried out under protection from light. The reading was held in an immunofluorescence microscope (NIKON - Eclipse) under 40x objective and ocular, 10x. The final was the highest dilution was considered positive according to the criteria of [32], this is with total peripheral fluorescence and homogeneous of tachyzoites.

Preparation of Supernatant: Preparation of supernatant soil samples, was carried out by concentrating the amount of *T. gondii* in soil sampling. For the water samples were used, 9 ml of sucrose solution with an initial density of 1.275 g/cm^3 for each 3 mL sample of water, then subjected to the centrifugation at 2500 rpm for 10 minutes. For soil samples a gram of soil was placed in a container and homogenized with 9 mL of saturated solution of sucrose with density 1.27 g/cm^3 and subjected to flotation technique second Ruiz *et al.* [33]. Then, the resulting product was transferred to conical tube with cap and subjected to centrifugation at 2500 rpm for 10 minutes [34]. The supernatant samples were designed for the mice bioassay and PCR.

Polymerase Chain Reaction (PCR) for Detection of *T. Gondii* DNA in Soil Samples: The supernatant samples of water and soil were centrifuged at 13000 rpm and genomic DNA was extracted using the commercial DNAzol Kit (Invitrogen®). The samples were stored at -20°C until the time of execution of the PCR. The amplification of *T. gondii* DNA was performed using the method described by Homan *et al.* [35]. *T. gondii*-specific primers pairs (gender) Tox4 and Tox5 (Tox 4: 5' CGC GAA GAC TGC AGG GAG GAA AGT TG 3' and Tox5: 5' CGC AGT CAC TGC AGA GCA TCT GGA TT 3') that amplify fragments of 529 bp [35] were employed.

The RH strain of *T. gondii* was used as a positive control for toxoplasmosis. Ultra-pure water was used for negative control. The limit of detection of DNA of *T. gondii* in soil samples was 2.9 x 10^6 tachyzoites per mL and 2.9 x 10^2 tachyzoites per mL.

The analysis of the amplified products were performed by electrophoresis on 1.0% agarose gel with TBE running buffer 0.5 X (0.045 M TRIS-borate and 1 mM EDTA pH 8.0) and the gel stained with ethidium bromide was subjected to constant voltage of 7.6 v/cm.

Bioassay in Mice: Gentamicin (2%) was added for each 1 mL supernatant sample and immediately collapsed in insulin syringes, which has become the inoculum for the mouse bioassay.

For the isolation of *T. gondii* from supernatant soil samples albino mice Swiss type were used. They were young adults, with weight, approximately 20 to 30 grams. They were divided into three groups (test, positive control and negative control). During the bioassay, the animals were kept in boxes polypropylene in the vivarium of the Laboratory of Rabies and Encephalitis of the Institute Biológico of São Paulo, with the alternate system of light, being 12 h of light and 12 h of darkness and exhaustion of air. The animals were fed with pelleted ration industrial and public water supply.

Five mice were used for each sample of innocuous (supernatant of the soil), two inoculated intraperitoneal (IP) and three by oral (VO) per route.

In total, for the bioassay were inoculated 20 mice IP with 0.2 mL of supernatant of the soil and 30 mice VO per routes that ingested 0.2 mL the supernatant sampling of the soil (Table 1 and 2).

Positive control group composed of five animals that were inoculated orally with sample cystogenic *T. gondii*, donated by the Institute of Tropical Medicine of São Paulo and another negative control group composed of three animals inoculated intraperitoneally and others three animals were inoculated orally with sucrose solution (128g/100ml) density 1.275 mg/cm^3.

Collection Samples of Mice

Serum: Serological studies were performed at 0 and 30 days post-inoculation of mice by puncture of the lateral caudal vein after topical anesthesia of lidocaine 2%. At 60 days, blood was collected by cardiac puncture and mice were both weighed in analytical and anesthetized with a solution of 2% xylazine hydrochloride and ketamine hydrochloride 10%, diluted 2mg/mL and 10mg/mL in saline (0.9%), respectively. The anesthetic was administered as a single dose of 0.1 ml per 10g body weight IP [36]. After the procedure of blood collection, the animals were euthanized in a CO_2 chamber.

Organs: After euthanasia, the mice were submitted to necropsy for ablation of organs - liver, lung, spleen, heart and brain - which were stored in conical tubes properly identified and preserved in freezer at -20°C until the time of execution of the PCR.

RIFI of the serum of mice: Serum samples from mice were subjected to RIFI for determining the presence of antibodies anti-*T. gondii*, following the method described by [32]. The serum was diluted at a ratio of 1:16, as described by Da Silva and Langoni [37], Kourenti *et al.* [38] and Costa-Silva and Pereira-Chioccola [39]. For staining of glass slides the conjugate anti-mouse IgG were

used, marked with fluorescein isothiocyanate (anti-mouse IgG- whole molecule -FITC, produced in goat, affinity isolated antibody - SIGMA F0257) diluted at 1:250 in a solution of Evans blue to 0.001. Serum samples that showed fluorescence under title 16, were considered reactive and were subjected to new RIFI in base 2 serial dilutions, followed the same protocol. The final was a higher dilution that was considered positive. This is, the presence of fluorescence total peripheral and homogeneous and morphologically compatible with *T. gondii*.

PCR for Detection of *T. Gondii* DNA in Organs of Mice: For detection of *T. gondii* DNA in mice organs, PCR was performed according to Garcia *et al.* [40]. A fragment of 0.2 g of each organ was macerated with 0.8 mL of Elution Buffer [(TE), (1.2 g Tris; 1 mL of EDTA 0.5 M, pH 8; 1 L MilliQ water, pH 7.4)] and maintained at -20°C.

Genomic DNA was extracted from organs using DNAzol (Invitrogen®). Initially the suspension of the samples was subjected to centrifugation 2000 rpm and 500 mL of the supernatant, transferred to new tubes, identified for centrifuged 13000 rpm for 20 minutes, discarded the supernatant by inversion and the sediment resuspended in 100 mL of TE. The following was added 1 mL of DNAzol and homogenised by inversion, centrifuged for 10000 rpm by 10 minutes. From the material centrifuged despised it the supernatant by inversion and were added of 500 mL of pure ethanol, homogenized sample and centrifuged by 4000 rpm by 2 minutes, discarded if the supernatant by inversion and were added 850 mL of ethanol at 75%, centrifuged for 4000 rpm for 2 minutes. Again, despised if the supernatant by inversion and were added 850mL of ethanol at 75% and centrifuged. The supernatant was discarded, or dropped, with the help of a pipette and then the sediment was added 100 mL of NaOH at 8 mM and 40 mL of HEPES solution 0.1 M. The samples were stored at -20°C until the time of execution of the PCR.

The amplification of *T. gondii* DNA was performed using the method described by Homan *et al.* [35]. The specific pairs of primers, Tox 4: 5' CGC GAA GAC TGC AGG GAG GAA AGT TG 3' and Tox5: 5' CGC AGT CAC TGC AGA GCA TCT GGA TT 3' that amplify fragments of 529 bp were used [35, 40]. For every two clinical specimens were used 19.25 mL of ultra pure water to dilute 8.0 mL of DNTP, 5.0 mL of buffer, 3.0 mL of primer Tox 4, 3.0 mL of primer Tox5, 1.5 mL $MgCl_2^+$ and 0.25 mL enzyme taq polymerase, for one volume final of 20 mL at be distributed in microtubes and following plus 5 mL of each clinic sample at be amplified. The amplification was performed in thermal cycler, starting with incubation at 94°C for 7 minutes followed by 35 cycles at 94°C for 1 minute (denaturation), 55°C for 1 minute (annealing), 72°C for 1 minute (extension) and 1 cycle at 72°C for 10 minutes for final extension.

The establishment of the detection threshold of PCR was carried out from the experimental infection of organs with healthy mice, strain of *T. gondii* Rh. The infectious dose was determined in a Neubauer chamber. The concentration of the initial suspension was 2.9 x 10^7 tachyzoites/mL and this suspension was removed 0.1 mL for contamination experimental, as from 0.9 mL of - lung, liver or brain - macerated individually and subjected to serial dilution of base 10. Each dilution of infected organ, was performed DNA extraction with the commercial reagent DNAzol (Invitrogen) using it the primers described by [35].

The detection limits was 2.9 x 10^6 tachyzoites/mL for the liver and was 2.9 x 10^3 tachyzoites/ mL for lung and brain.

A strain of *T. gondii* cystogenic we used as a positive control for toxoplasmosis, while solution of clinical samples was used for negative control.

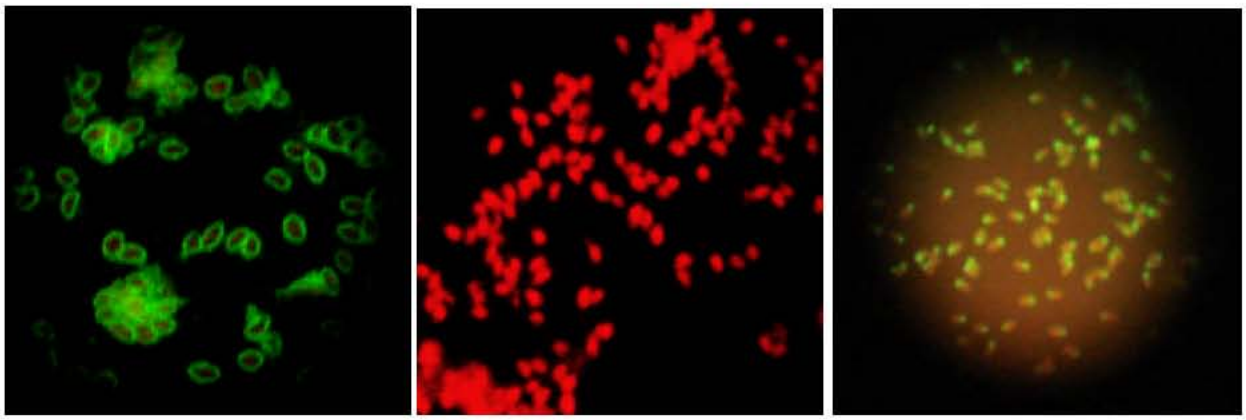
The amplified product were homogenized 9 mL of the samples amplified in 1 mL dye with glycerin (Blue Juice - Invitrogen®) and in following was performed by electrophoresis in agarose gel at 1% (3g agarose at be diluted with 30 mL of TBE (10X) - 107g of Tris base, 55g boric acid, 7.44 g EDTA (tristriplex) and add in 270ml of MilliQ water, 3.0 mL of bromide ethidium) in running buffer TBE [10x] (100ml TBE [10x] in 1000 mL of distilled water q.s.p) in constant voltage of 136V by 30 minutes and posteriorly photographed under ultraviolet light (300-320nm) by photo-documentation system (Cannon camera) and analyzed with the software D Image Analysis.

Statistical Treatment of Data: To calculate the frequency of anti-*T. gondii* in sheep herds serum-reactive to the RIFI and the proportion of positives to PCR and RIFI between the of different groups of mice subjected to bioassay (IP and VO routes), were utilized the Chi-square or Fisher exact test [41] with a significance level of 5%.

RESULTS

The four ruminants herds were serum-reactive at RIFI, with antibodies titers ranging from 1:64 to 1:1024. Antibodies anti-*T. gondii* were detected in 31.62% of ovine (86/272) and 94.12% (16/17) of cattle analyzed by RIFI.

It was observed that the four farms showed oocysts in supernatant samples of the soil (Tables 1 and 2; Figure 1).



(A) – Positive standard (B) – Negative standard (C) – Negative Apical standard

Fig. 1: Indirect immunofluorescence reaction - RIFI (magnification of 400X): (A) - Positive standard; (B) - Negative standard; (C) - Negative Apical standard

The experimental study by inoculating suspension of supernatant for the samples of soil showed higher mean overall 70% (14/20) of mice inoculated intraperitoneally (IP) reagents versus 63.33% (19/30) of mice inoculated orally (VO) reagents to RIFI.

Means relatively high of anti-*T.gondii* of antibodies titers by RIFI in mice inoculated by IP route with supernatants extracted from soil collected from various sites ranged from 50% (site B) and 83.33% (site A). For animals inoculated in the IP, after 30 days (30 dpi), 60% of mice were reactive and had titers ranging from 1:16 to 1:256 and 50% of mice were positive and after 60 days showed titers ranging from 1: 16 to 1:128. Similarly, relatively high average of anti-*T. gondii* titers of antibodies by RIFI in mice inoculated with supernatants by VO route extracted from soil collected from various sites ranged from 50% (site B and C) to 88.89% (site D). For animals inoculated in the VO routes, after 30 days (30 dpi), 43.33% of mice were reactive with titers between 1:16 to 1:32 and 40% after 60 days of mice had titers equal to 1:16. Such variations of values among the IP and VO routes of inoculation were not significantly ($P>0.05$) different (Table 1).

In the present research, the figure 1-A showed positive serological sample where fluorescence total peripheral and homogeneous was observed and figures 1-B and 1-C presented serums with fluorescence incomplete, apical or absent apical, which were considered negative.

The experimental study conducted by inoculating suspension of supernatant of soil samples showed higher mean overall 30% (6/20) of mice inoculated IP positives versus 23.33% (19/30) of mice inoculated VO reagents to PCR.

For mice inoculated by different routes with supernatant samples extracted from soil samples from different sites (A, B, C and D) in sheep-farms of sheep and sheep associated with cattle, the positivities of the different organs of mice were 30% in lung (IP) and 16.67% in lung (VO), 3.33% in liver (VO) and 3.33% in brain (VO). Like variations of percentages between the means of the various organs of mice submitted IP and VO inoculation routes and diagnosed by PCR were not significantly ($P>0.05$) different (Table 2).

DISCUSSION

The analysis of the interaction between conditions of farms, which are intended to grazing herds, demonstrated a close relationship between the eco-epidemiology of protozoa of toxoplasmosis and the percentage of reacting animals when 31.62% of ovines and 94.12% of bovines were serum-reactive at RIFI, with antibodies titers ranging from 1:64 to 1:1024. The four farms showed oocysts in the soil samples and the experimental study. Higher mean overall 70% of mice inoculated IP reactives versus 63.33% of mice inoculated VO reagents to RIFI were recorded (Table 1 and 2; Figure 1). So overall, the main risk factor that significantly increased the frequency of animals and herds reagents with a high incidence of serum reagents was the influence of eco-epidemiology, marked by the coexistence of sheep with felines (cats) in the sheep-farms. In these farms was observed that the felines has by habit after defecation, cover the faeces with soil in the various areas for grazing animals.

According to Ferguson [42], arguably, domestic cats are definitive hosts of toxoplasmosis in enabling the development of the life cycle of asexual and sexual and therefore the spread and permanence of the parasite in the environment. The above author affirmed that infections can result in one of ways. The first, or rarest way, is congenital transmission. It occurs if a female (human or animal) becomes infected for the first time during pregnancy with the circulating tachyzoites associated with the acute phase crossing the placenta to infect the fetus. The second was from the ingestion of sporulated oocysts resulting from the contamination of food or water with cat faeces and would explain the incidence in herbivores. The third when the intermediate host (bird or mammal) is ingested by the definitive host of toxoplasmosis.

Results similar to this research were presented by Da Silva *et al*. [24] registered in 173 serum sample of 67.6% of sheep positive in the Zona of Mata and 32.4% of sheep serum reagents by RIFI in the region of Agreste, both in the Brazil. For this high prevalence of toxoplasmosis, moisture and vegetation type contributed to the formation of a favorable microenvironment for the survival rates of oocysts in the soil.

In the present study, evidence of high soil contamination by oocysts of toxoplasmosis can be established through all groups of inoculated mice which showed average increases of anti-*T. gondii* by RIFI titers of antibodies in mice inoculated by IP route collected from various locations of the farms, ranging from 50% (site B) 83.33% (site A). Still, it may be noted that 60% of mice were reactive (30 dpi) with titers ranging from 1:16 to 1:256 and 50% of mice were reactive (60 dpi) with titers ranging from 1:16 to 1:128 (Table 1).

Similar results were observed for VO route of inoculation. Mean anti-*T. gondii* titers of antibodies by RIFI in mice inoculated with soil samples ranged from 50% (B and C) to 88.89% (site D). At 30 dpi, 43.33% of mice were reactive with titers between 1:16 to 1:32 and at 60 dpi, 40% of mice showed evidence of decreasing titers equal to 1:16 (Table 1).

The eco-epidemiological studies of the farms revealed that the farm A showed intense promiscuity with cats.

Though soil samples of the four farms had oocysts (Tables 1). However, was not observed equivalence between the frequencies of animal's serum reagents. Of these observation, could be inferred that the viability of oocysts in the environment would be compromised, which may restrict the dissemination of the agent between animals. On the other hand, too must be emphasized the important role of disseminator feline, present on the farm-A.

Regarding the capacity of IP and VO routes of inoculation of the soil samples could be inferred at least one hypothesis: the infective dose of oocysts present in soil samples of the farm-A, would be able to produce infection in mice bioassay, regardless of inoculation IP or VO route. Reverse fact, was observed for water samples from this farms, but only the IP route was significantly more infectious than VO. In farm-A, its environmental conditions and sloping topography, besides allowing the sheep access to contaminated soil by cat feces, were aggravated by a predisposition to leaching from the soil by rain water toward the lake where the animals were supplied with drinking water, favoring the dissemination of infection in this farm (Tables 1 and 2).

However, two important risk factors should be considered in this investigation, to explain the possible presence of *T. gondii* in the four herds of sheep-farms: first, due to the constant presence of feline, domestic cat in farms; and the second is due to access free animal surface waters, resulting of the increase of leaching the soil by rain and carried for the water drinkers of ground floor and were always available for watering of herds of animals.

Just as in this present research, among the studies that reported results for both sheep and cattle, Garcia *et al*. [22] in farms of North of Parana determined 51.8% (118/228) serum samples from sheep and 25.8% (103/400) samples of cattle reagents by RIFI and Meireles [43] to study the prevalence of toxoplasmosis in 200 sheep of the municipality of San Manuel and 200 cattle in Taquarituba, of São Paulo state, determined 11% and 31% of cattle and sheep, respectively, reagents by ELISA technique.

The importance of reflexes of the high incidence of contamination with oocysts or infective forms of *T. gondii*, this is, source of origin felines, farm animals acting as intermediate hosts on the dissemination in susceptible livestock, was demonstrated in this research, through bioassay inoculation suspension of soil, resulting from the grouping samples from these sites, which showed overall average of 70% of mice inoculated intraperitoneally (IP) versus 63.33% of mice inoculated VO, reagents RIFI. All the properties have responded positively to inoculation via IP and VO in mice. Despite of the means, IP and VO routes, show whether different, however, this difference ($P>0.05$) results of environmental

contamination when tested by bioassay for the 30 dpi and 60 dpi in the various studied farms, proved to be high potential environmental contagion for both tests independent of the route (Table 2).

The sampling to bioassay, showed the higher titers that went up until 1:256 for 60% (30 dpi) and 50% (60 dpi) of mice inoculated via IP versus titers that went up until 1:32 to 43.33% (30 dpi) and 40% (60 dpi) from mice inoculated via VO (Table 2).

There is still no reliable method for large scale use in isolation of *T. gondii* oocysts from soil samples [44]. Although it is difficult to achievement the mice bioassay it is more reliable until the present. Thus, in agreement with the results of the present research, Ruiz and Frenkel [45], detected by the microscopic oocysts in 12.7% of cat feces samples, while that at bioassay, 87.3% of the mice.

Domestic cats play an important role in the epidemiology of toxoplasmosis [46], since, contaminating water, soil and pasture, with oocysts eliminated by their faeces [47, 12, 13] can therefore be said that the oocysts eliminated, resulting in environmental contamination [44].

Infection rates in cats, are usually determined by the rates of infection in populations of birds and rodents, which serve as food source [44]. In the investigation epidemiological, data of seroprevalence in cats are more common than fecal examinations, since the presence of antibodies in cats indicates that probably already have eliminated oocysts and therefore, can be considered as indicators of environmental contamination [48].

After ingestion of tissue cysts by cats, the cyst wall is dissolved by proteolytic enzymes of the stomach and small intestine. The released bradyzoites penetrate the epithelial cells of the intestinal wall and initiate numerous asexual multiplications of generation of *T. gondii* [7].

The congenital transmission between sheep and lambs, often constitute a cause of abortion [6, 15, 50] and death during fetal development, birth and development of lambs rarely occur [15], there is still the possibility of occurrence of mummified fetuses [51].

Pena *et al.* [52] analyzed the serum of 237 cats of São Paulo state, coming from 15 cities, being that 35.4% of cats errants were reagents for *T. gondii* antibodies by modified agglutination test (MAT - modified agglutination test).

Non-sporulated oocysts can survive more than 11 weeks at refrigerator temperature. In the environment, can survive for less than 3 months with a capacity of becoming infectious [49]. The oocysts of *T. gondii* may persist in the environment for a long time [53].

The survival of oocysts sporulated in water at a temperature of -10°C to 70°C for different periods was studied by Dubey *et al.* [7] and infectivity was confirmed by bioassay in mice, with no loss of infectivity when stored at temperatures of 10°C, 15°C, 20°C and 25°C for 200 days, 30°C for 107 days, 35°C for 32 days at 40°C for 9 days, 45°C for 1 day for 1 hour at 50°C, 55°C and 60°C in 2 and 1 minute, respectively. Still survives at temperatures of 4°C for 54 months and the temperature of -5°C for 106 days and -10°C for 13 months.

The horizontal transmission of *T. gondii* may involve three stages of its life cycle: the ingestion of oocysts from the environment and ingestion of tachyzoites contained in meat or guts of different animals [6].

Matsuo *et al.* [56] proposed treatment of the sediment resulting from centrifugation of soil samples contaminated experimentally with oocysts of *T. gondii* in flotation sucrose solution plus 0.1% gelatin, using if 1-polyvinylpyrrolidone (PVP) added to TE buffer, heating and cooling (at 98°C for 10 minutes followed by 4°C for 10 minutes), of samples followed by the addition of 100 mM NaCl and the addition of bovine serum albumin (BSA) during amplification and these changes proposals contributed to the removal of PCR inhibitors in samples of soil and consequently the increase of the sensitivity of the test.

In the present study, could not rely on changes in the sucrose flotation technique and the use of procedures to remove PCR inhibitors, as described by [56].

The diagnostic by PCR, in the experimental study by inoculating suspension of supernatant for the sampling of soil showed higher mean overall 30% (IP) and 23.33% (VO) of mice reagents. The higher positivities in the tissues of mice ranged of - lung (IP: 30%; VO: 16.67%), liver (VO: 3.337%) and brain (VO: 3.33%) - of mice inoculated by different routes with sampling of supernatants extracted from soil samples from different sites farms (A, B, C and D). Results showed that PCR was more efficient in detecting reagents mice to the pathogen in tissue samples of animals that had contact both by the IP as VO routes with oocysts of *T. gondii*, when the sampling came from local soil, being the higher incidence of reagents, in decreasing order of election, the tissues of lung, liver and brain (Table 2).

The bioassay in mice is the standard test for detecting infection in tissues [35], due to its high sensitivity and specificity [40], however, is laborious, costly, time consuming and offers risks to the operator [57].

The promiscuity of the domestic cat with the sheep herd of the farm-A, in which the faeces were deposited in the soil of grazing animals, was the principal predisposing factor. The positivity was confirmed by soil sampling to bioassay and formed in strong evidence of predisposition to soil contamination by oocysts and consequently to dissemination of toxoplasmosis between the animals.

The polymerase chain reaction (PCR) is widely used for detecting *T. gondii* in amniotic fluid, blood and body tissue [8], as well as in water samples [18, 53, 58, 38] and soil [56].

Likewise, to Kourenti *et al.* [38], the presence of oocysts and *T. gondii* DNA in water is coming from of wells, is evidence of environmental contamination and dispersed forms of the parasite may create a risk of toxoplasmosis transmission between humans and animals.

However, Villena *et al.* [53], described, strategies for detection of many parasites in the water, including oocysts of *T. gondii*, through of amplification by polymerase chain reaction, which was able to detect *Toxoplasma* DNA samples, where the mice bioassay were negative, mainly with relation the application of sucrose flotation.

Sroka *et al.* [58], examined 114 samples of water at Loblin region (east of Poland) the presence of *T. gondii* DNA that was determined as positive in 27.2% (31/114) of samples, being 30 of shallow well and a deep well, whereas all samples of water supply system were negative.

Results of bioassay, similar to this research were presented by Dubey *et al.* [59] through of test in mice detected strains isolated from birds with different responses and the genotype 3 strain showed tropism for the lung for the death of mice from 13 days after inoculation of tissue cysts of lory.

Even if the PCR technique may be employed in various diagnostic procedures due to its high sensitivity and specificity, in present research, it was not possible to detect the presence of DNA from oocysts of soil in four farms investigated. Limitations of the technique resulting in inhibitory substances, of the process of extracting DNA from environmental samples or other factors could explain these results. The sensitivity of PCR can be affected by improper handling, sending and storage conditions of samples [8]. Likewise, in studies of [37] to compare different methods for diagnosis of toxoplasmosis, had affirmed that it was possible to detect toxoplasmosis in tissues of brain and diaphragm in 7.7% (40/522) and titers ranging from 1:16 to 1:1024 sheep herd, when percentages of them had been negative by RIFI. The percentage was of 66.7% (26/39) and 53.8% (21/39) in brains of mice digested by two methods and the percentage of positive PCR was 17.9% in brain and 9% in diaphragm.

CONCLUSION

The herds of exclusive sheep and sheep associated with cattle showed percentages of reagents relatively high and ergo the anti- *T. gondii* titers of antibodies were worrying. The study of eco-epidemiology through the soil samples, animal serum and the mouse bioassay tests with samples from the four farms studied, showed significant effects of interaction between definitive host and survival of oocysts in soil and subsequent infection of animals. Aggravating fact, the possibility of dissemination of protozoonosis, via contamination of soil, was the constatation of the promiscuity of the herds with the definitive host, the domestic felines. Was possible to show the frequencies of experimental materials and animals reagents at protozoonosis *T. gondii* detected by RIFI, PCR and to bioassay. For analysis of soil farms studied at bioassay for different routes of inoculation (IP or VO), was more efficient than the PCR performed in supernatants of soil samples. The lungs of mice, proved to be the organ of choice after studying the bioassay and detection of *T. gondii* at PCR; All samples of soil analysis by PCR technique were negative; and established the complexity and importance of theme in the study of eco-epidemiological relation to frequency of animals reacting to the *T. gondii* by the techniques of isolation in mice, RIFI and PCR would be very important to complement these data with future studies initiated in the theme of results for expansion and improvement of experimental data.

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