

Infection by *Toxoplasma Gondii* in Herds of Sheep-farming Exclusive and Intercropping with Cattle and the Environmental Contamination by Oocysts

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Abstract: This study aimed to determine the toxoplasmosis in sheep and cattle herds in the region of Sorocaba-SP and to prove the presence of oocysts in samples of water through of mice bioassay and PCR. Were studied 272 sheep and 17 bovines from four properties in the region of Sorocaba. The immunofluorescence indirect reaction (RIFI) for the research of anti-*Toxoplasma gondii* antibodies was performed. The research of the presence of oocysts of *T. gondii* in samples of surface waters (drinking water) was carried out through bioassay in mice inoculated intraperitoneally (IP) and orally (VO). In days 0, 30 and 60 after inoculation were harvested and from the blood serum samples mice analyzed for observe the seroconversion by RIFI. After 60 days of inoculation, the mice were euthanized and organs analyzed by polymerase chain reaction (PCR). Water samples were analyzed for the purpose of detecting the DNA of *T. gondii* by PCR. The positivity in herds was of 31.62% sheep serum-reagents, and 94.12% of bovine serum-reagents at RIFI. The reactivity in the bioassay was of 57.69% (IP) and 43.59% (VO) of mice serum-reagents at RIFI. The positivity of organs of mice was of 57.69% (IP) at PCR. All samples of water analyzed by technique of PCR, were negative. The results of the experimental conditions, can concluded if that toxoplasmosis is present in all four herds sheep examined of the region of Sorocaba. The contaminated water, proved by mice inoculation (bioassay) was considered important sources of the infection.

Key words: *Toxoplasma gondii*, Oocysts, Sheep, Bovine, Water

INTRODUCTION

Of agreement to the latest Census of Agriculture conducted in 2006, in Brazil were estimated at 13,9 million of sheep and 169,9 million of cattle (IBGE 2006). The consolidation of the productive chain of the sheep industry in Brazil was marked by changes in the last ten years. However, among other limiting factors, it stands out the deficiency of effective sanitary control centers in farms (Medeiros *et al.* 2005; Carvalho 2008). Accurate diagnostic is essential for any parasitic approach sanitary in sheep and goats, otherwise the creation of these species becomes unviable due to low productivity (Jardim 1996).

Toxoplasma gondii, protozoan that present facultative life cycle heterogeneous, and has the felids as the definitive host, while the other species of mammals and birds act as intermediary hosts. It is a parasite with an important pathogenic role in fetal medicine and their classification is: Domain (superkingdom): Eukaryote; kingdom: Alveolata; Phylum: Apicomplexa; Class: Coccidia; Order: Eucoccidiorida; Family: *Sarcocystidae*; Genus: *Toxoplasma*; Specie: *Toxoplasma gondii* (Nicolle and Manceaux 1908). The Toxoplasmosis, severe infirmity that affects many species and can cause embryonic death and resorption, fetal death and mummification, abortion, neonatal death, mainly in sheep (Freyre *et al.* 1999) and goats (Dubey 2004).

In the animals, the toxoplasmosis is related to the ingestion of oocysts present in food and water contaminated (Plant *et al.* 1974; Coutinho *et al.* 1982), however, is the ingestion of oocysts as the principal

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route of transmission for herbivores, mainly sheep and goats (Engeland *et al.* 1996; Duncanson *et al.* 2001). The outbreaks of toxoplasmosis in humans may be related to environmental contamination by oocysts (Tenter *et al.* 2000), or by a habit of certain ethnic groups, relative to the handling and consumption of meat or offal from sheep, raw or under-cooked (Larsson *et al.* 1980). 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 (Spósito-Filha 1992).

To Isaac-Renton *et al.* (1998), should also consider the possibility of the dissemination of non-sporulated oocysts through surface water. 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 to be identified sexual stages in the small intestine of cats (entero-epithelial cycle) and oocysts in their feces (Frenkel *et al.* 1970).

In serology of sheep and cattle, important studies have been applied to research of herds reagents *T. gondii* by RIFI, being the frequencies found in: sheep: 34.7% (207/597) (Figliuolo *et al.* 2004) and 54.6% (185/339) (Ogawa *et al.* 2003) and in dairy cattle of: 48.51% (Marana *et al.* 1995). In the Oregon, United States, toxoplasmosis was diagnosed as third cause infectious and parasitic of abortion, and perinatal mortality in sheep (Dubey and Kirkbride 1989).

In the present research, carried in sheep farms exclusive, and sheep farms in intercropping with cattle grazing from the region of Sorocaba, Sao Paulo state, had the following objectives: to determine the incidence *T. gondii* in sheep of antibodies to and cattle by indirect immunofluorescence reaction (RIFI); to detect *T. gondii* DNA by polymerase chain reaction (PCR) in samples of surface water, water drinker; and to analyze of presence of *T. gondii* oocysts in samples of surface water and water drinker through of the bioassay method in mice by RIFI and PCR.

MATERIALS AND METHODS

Four herds originating from the region of Sorocaba, Sao Paulo state, were studied: two of sheep farm exclusive, and two sheep farms in consortium in grazing with cattle. The herds, for the production of meat, were predominantly composed of females and kept in semi-extensive system with the use of grazing, supplemented with commercial feed, salt in trough, and made use of drinking water through access the surface waters such as rivers streams and springs, or received the water in water drinker.

To determine the presence of *T. gondii* in conditions of the sheep-farm, the four herds investigated were to two risk factors: the living promiscuity of animals with domestic cats and free access to the waters surface. The clinical and environmental samples were collected in the period from February to March 2008.

The serum of sheeps and bovines were subjected to Indirect Immunofluorescence Reaction (RIFI), for determining the presence of *T. gondii* antibodies according as Camargo (1974) were diluted in buffered saline (PBS), pH 7.4 in the proportion of serum 1 μ L to 64 μ L have PBS. Were placed on blades previously sensitized with tachyzoites of *T. gondii* RH strain stained, using conjugated sheep anti-IgG, labelled with fluorescein isothiocyanate (affinity Purified Antibody Fluorescein rabbit 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 rabbit 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 a 40X objective and ocular, 10X. The final was the highest dilution was considered positive according to the criteria of Camargo(1974), this is, with total peripheral fluorescence and homogeneous of tachyzoites.

Preparation of supernatant samples of water, was carried out with the, purpose of concentrating the amount of *T. gondii* in samples of water. For the water samples were used, 9 ml of sucrose solution with an initial density of 1.275 g/cm³ for each 3 mL sample of water, then subjected to the centrifugation at 2500 rpm for 10 minutes. Then, the resulting product was transferred to conical tube with cap and subjected to centrifugation at 2500 rpm for 10 minutes (Azevedo 1981). The supernatant samples were designed the mice bioassay and PCR.

Polymerase Chain Reaction (PCR) for DNA Detection of T. gondii in Water Sampling:

Genomic DNA was extracted from samples of the supernatant of water 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.* (2000). *T. gondii*-specific

primers 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 were employed (Homan *et al.* 2000).

As a positive control for toxoplasmosis was used the Rh strain of *T. gondii*. For negative control was used ultra-pure water. The limit of detection of DNA of *T. gondii* in water samples was 2.9×10^2 tachyzoites per mL.

For the analysis of the amplified products were performed by electrophoresis on agarose gel the 1.0% 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 Mices:

For each one mL supernatant sample was added 2% gentamicin and immediately collapsed in insulin syringes, which has become the inoculum for the mouse bioassay. For the isolation of *T. gondii* from supernatant samples of water were used albino mice, Swiss type, young adults, with weight, approximately 20 to 30 grams, 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, 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.

For each sample of innocuous (supernatant water) were used five mices, being two inoculated intraperitoneal per route and three by oral route. In total 26 mices were used for the bioassay of the supernatant of water sampling. The animals were inoculated intraperitoneal per route with 0.2 mL supernatant of water sample. For the inoculation by the oral route were used 39 animals that ingested 0.2 ml of supernatant samples of water.

During the experiments was preserved a positive control group composed of five animals that were inoculated orally with sample *T. gondii* cystogenic, donated by the Institute de Medicina Tropical de São Paulo, and another negative control group composed of three animals inoculated intraperitoneally, and three animals were inoculated orally with sucrose solution (128 g/100 mL) density 1.275 g/cm^3 .

The present work was evaluated and approved by the Ethics Committee in Animal Experimentation - CETEA-IB, identified by protocol number 47/08, in 16 April of 2008 and is in accordance with the Ethical Principles in Animal Experimentation adopted the Brazilian College of Animal Experimentation (COBEA).

Collection Samples of Mice:

Serum:

The serology sampling by puncture of the lateral caudal vein after topical anesthesia of lidocaine 2%, were performed at 0 and 30 days post-inoculation of mice. 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.

Organs:

After euthanasia, the mice were submitted to necropsy for ablation of organs the liver, lung, spleen, heart and brain, which were stored in conical tubes properly identified and maintained in freezer at $-20 \text{ }^\circ\text{C}$ until the time of execution of the PCR.

Indirect Immunofluorescence Reaction (RIFI) of the serum of mice: Serum samples from mice were subjected to RIFI for determining the presence of anti-*T. gondii* antibodies, following the method described by Camargo (1974). The serum was diluted at a ratio of 1:16, as described by Da Silva *et al* (2001), Kourentii *et al* (2003), Costa-Silva and Pereira-Chioccola (2010). For staining of glass-slide were used the conjugate anti-mouse IgG, 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.

Polymerase Chain Reaction (PCR) for Detection of DNA of *T. gondii* in Organs of Mice:

For DNA detection of *T. gondii* in organs of mice of the bioassay was performed by the polymerase chain reaction (PCR). An aliquot 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 $-20 \text{ }^\circ\text{C}$.

Genomic DNA was extracted from organs using DNAzol (Invitrogen®). Initially the suspension of the samples was subjected to centrifugation 2000 rotations per minute (rpm) and 500 mL of the supernatant formed, transferred to new tubes, identified for centrifuged 13,000 rpm for 20 minutes, discarded the

supernatant by inversion, and the sediment resuspended in 100 mL of TE. Again, despised if the supernatant by inversion and were added 850 mL of ethanol at 75% and centrifuged. The supernatant was discarded, or dropped, with the help of a pipette, 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*²³. Were employed gender specific primers 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 (Homan *et al.* 2000; Garcia *et al.* 2006).

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 10⁷ tachyzoites/ mL and this suspension was removed 0.1 mL, for contamination experimental, as from 0.9 mL of lung, liver and 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 Homan *et al* (2000).

For the liver, the detection limits was 2.9 x 10⁶ tachyzoites/mL and 2.9 x 10³ tachyzoites/mL for lung and brain. As a positive control for toxoplasmosis, we used a strain of *T. gondii* cystogenic, and for negative control, was used diluted solution of clinical samples.

For analysis of the amplified product, were homogenized 9 mL of the samples amplified in one mL dye with glycerin (Blue Juice - Invitrogen^o), 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.

In Statistical treatment of data, to calculate the frequency of anti-*T. gondii* antibodies in sheep in the region of Sorocaba, SP, were counted the number of animal serum-reactive to the RIFI on the total number of animals examined. Comparing the different groups of mice subjected to bioassay the proportion of positives, was used the chi-square or Fisher exact test (Callegari-Jacques 2003), with a significance level of 5%.

Results:

Table 1: Frequency of herds and animals for anti-*T. gondii* antibodies in the indirect immunofluorescence reaction (RIFI) in exclusive of sheep farm, and sheep farm in consortium with cattle.

Identification of property	Conditions of farm	Reagents		
		Herds	Reagents Animals/ examined total (%)	
			Sheeps	Bovines
A	O	2/2	28/37 (75.67)	-
B	O		20/91 (21.98)	-
C	C	2/2	10/37 (27.03)	3/3 (100)
D	C		28/107 (26.17)	13/14 (92.86)
Total		100%	86/272 (31.62)	16/17 (94.12)

O - Sheep farms exclusive; C - sheep farms in consortium with bovine

In farms exclusive of sheep, 75.67% (herd A) and 21.98% (herd B) had anti-*T. gondii* antibodies by RIFI. The overall mean was of 31.62% for the four herds of sheep that had *T. gondii* serum antibodies titers by RIFI (Table 1).

The anti-*T. gondii* of antibodies titers in herds (A and B) ovine ranged from 1:64 to 1:1024. The higher frequencies of reagents animals were 60% (B) and 43% (A) showing titers of 1:64, followed of decrease to 29% (A) and 15% (B) with a titer of 1:128, 25% (A) and 15% (B) with a titer of 1:256, 3% (A) and 5% (B) with titer of 1:512 and 5% (B) with titers of 1:1024 (Figure 1).

In the sheep farms intercropped with cattle, 27.03% (sheep herd C), 26.17% (sheep herd D), 100% (cattle herd C) and 92.86% (cattle herd D) had *T. gondii* antibodies by RIFI. The overall mean was 94% for the two herds of cattle with *T. gondii* antibodies by RIFI (Table 1).

The serum anti-*T. gondii* of antibodies titers in herds (C and D) of sheep associated with cattle ranged from 1:64 to 1:512. The higher frequencies of reagents animals and titers, respectively, were of 67% and 1:64, 1:128 and 50% (both in C bovines) and 43% and 1:512 (D ovines), followed by decreases and animal reagents in all herds with titers of 1:128, 1:256, and only 11% of reagents titers of 1:1024 sheep herd D (Figure 2).

Table 2: 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 surface water, collected in properties with sheep farms exclusive, and sheep farms associated with cattle.

Sheep-farm/site of collect	n.º of mices pos/inoculated	IP Titer		n.º of mices pos/inoculated	VO Titer	
		30 dpi ¹	60 dpi		30 dpi	60 dpi
A/ Water drinker	2/2	16; 16	32; NR	1/3	2 NR; 16	3 NR
A/ Lake	2/2	16; 128	32; 32	0/3	3 NR	3 NR
A/ Well water	½	NR; 256	NR; 16	0/3	3 NR	3 NR
Subtotal	5/6 (83.33 %)			1/9 (11.11%)		
B/ Water drinker	½	NR; 128	NR; 16	2/3	2 NR	NR; 16; 16
B/ Waterlogged	0/2	2 NR	2 NR	2/3	NR; 16; 16	3 NR
B/ Water fount	2/2	NR; 16	16; 16	2/3	NR; 16; 16	3 NR
Subtotal	3/6 (50.00%)			6/9 (66.67%)		
C/ Water drinker	½	NR; 64	NR; 16	1/3	2 NR; 16	2 NR; 16
C/ Lake	2/2	16; 16	32; 128	2/3	NR; 16; 16	NR; 16; 16
C/ Well water	½	NR; 16	NR; 64	0/3	3 NR	3 NR
Subtotal	4/6 (66.67%)			3/9 (33.33%)		
D/ Water drinker	½	NR; 16	16; 64	2/3	NR; 16; 32	2 NR; 16
D/ Lake 1	0/2	2 NR	2 NR	1/3	2 NR; 16	2 NR; 16
D/ Lake 2	0/2	2 NR	2 NR	2/3	2 NR; 16	2 NR; 16
D/ Water fount	2/2	16; 16	16; 32	2/3	NR; 16; 16	2 NR; 16
Subtotal; [range titer total]*	3/8 (37.50%)	14/26 [(53.85%);	15/26 [(57.69%);	7/12 (58.33%)	15/39 [(38.46%);	11/39 [(28.20%);
Total	15/26(57.69%)	titters: 16 – 256]	titters: 16 – 128]	17/39 (43.59%)	titters: 16 – 32]	titters: 16]

* The percentage of RIFI titers serum reagents from mice inoculated with samples of surface water after 30 and 60 days post inoculation (dpi), differed (P <0.05) significantly to the totals response between intraperitoneal (IP) and oral (VO) routes, only for the property A compared with other analyzed properties.
¹dpi: days post inoculation; NR: Not reagent; POS: Positive.

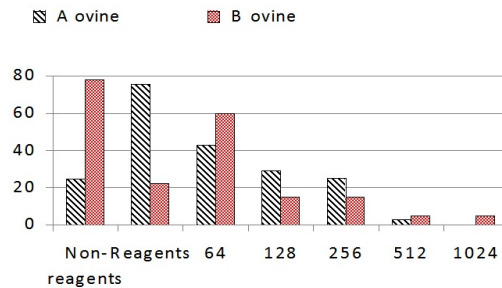


Fig. 1: Frequency of reagents animals, and distribution of anti-*T. gondii* of antibodies titers in two herds of sheep in consorting with cattle. NR: Non-Reagents; R: Reagents.

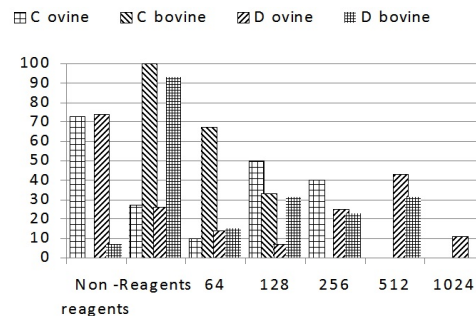


Fig. 2: Frequency of reacting animals and distribution of *T. gondii* of antibodies titers in two herds of sheep farm exclusive. NR: Non-Reagents; R: Reagents.

In the analysis of table 2 were observed that the four properties showed oocysts in samples of surface water and water drinker. The experimental study by inoculating suspension of surface water for the grouping of water sampling showed higher mean overall 57.69% (15/26) of mice inoculated intraperitoneally (IP) reagents RIFI versus 43.59 % (17/39) of mice inoculated orally (VO). However, only the mean of 11.11% of the sampling properties of group A at 30 dpi and 60 dpi showed difference (P<0.05) significantly between IP and VO routes, in relationship other properties for mices reagents to RIFI (Table 2).

In bioassay, the higher means of 53.85% [(14/26 IP; titters: 16 - 256)], and 57.69% [(15/26 IP; titters: 16 - 128)] of titers in mice inoculated via IP versus 38.46% [(15/39 VO; titters: 16 - 32)] and 28.20% [(11/39 VO; titters: 16)] of titers in mice inoculated VO routes (Table 2).

The experimental study by inoculating suspension of surface water for the clustering of sampling, revealed mean significantly (P<0.05) higher for 57.69% (15/26) of mice inoculated intraperitoneal (IP) route, were reagents to PCR versus 2.56% (1/39) of mice inoculated oral (VO) route. Among the three organs - lung, liver and brain - analyzed by PCR, 57% (15/26) samples of mice were positive for the lung of animals inoculated IP route and only 2.50% (1/39) of the lungs of mice VO inoculated via, were positive to PCR (Table 3).

Table 3: Diagnostic by PCR for the presence of *T. gondii* in organs of mice inoculated bioassay by intraperitoneal (IP) and oral (VO) routes with samples of surface water of draker.

Sheep-farm/site of collect	Mices ¹ POS/INOC	Intraperitoneal route (IP)			Mices POS/INOC	Oral route (VO)		
		PCR in the tissues studied				PCR in the tissues studied		
		N° POS / N° NEG				N° POS / N° NEG		
		Lung	Liver	Brain		Lung	Liver	Brain
A/ Water drinker	2/2	2 P	2 N	2 N	0/3	3 N	3 N	3 N
A/ Lake	2/2	2 P	2 N	2 N	0/3	3 N	3 N	3 N
A/ Well water	1/2	1 P / 1 N	2 N	2 N	0/3	3 N	3 N	3 N
B/ Water drinker	0/2	2 N	2 N	2 N	0/3	3 N	3 N	3 N
B/ Waterlogged	0/2	2 N	2 N	2 N	0/3	3 N	3 N	3 N
B/ Water of nascent	2/2	2 P	2 N	2 N	0/3	3 N	3 N	3 N
C/ Water drinker	1/2	1 P / 1 N	2 N	2 N	0/3	3 N	3 N	3 N
C/ Lake	2/2	2 P	2 N	2 N	0/3	3 N	3 N	3 N
C/ Well water	1/2	1 P / 1 N	2 N	2 N	0/3	3 N	3 N	3 N
D/ Water drinker	2/2	2 P	2 N	2 N	1/3	1 P/ 2N	3 N	3 N
D/ Lake 1	0/2	2 N	2 N	2 N	0/3	3 N	3 N	3 N
D/ Lake 2	0/2	2 N	2 N	2 N	0/3	3 N	3 N	3 N
D/ Water of nascent	2/2	2 P	2 N	2 N	0/3	3 N	3 N	3 N
Total (site): 13	15 P / 26 (57.69% P)*	15 P / 11 N (57.69% P)	26 N (100% N)	26 N (100% N)	1 P / 39 (2.56% P)	1 P / 39 (2.56% P)	39 N (100% N)	39 N (100% N)

* The percentage of positive (P) to PCR from tissues of the lung, liver and brain of mice inoculated with samples of surface water differ (P<0.05) significantly to the totals of response between intraperitoneal (IP) and oral (VO) routes.

¹N° mices POS/INOC: number of positive for the total mice inoculated, P: positive, N: negative.

Discussion:

The RIFI for serological detection of antibodies anti-*T. gondii* in herds (A and B) of sheep and herds (C and D) of sheep in consortium with bovine showed significant frequencies of serum reagents animals with a general mean of 31.62% for sheep and 94.12% for cattle herds from four farms in the region of Sorocaba in the state of São Paulo, studied in present research (Table 1).

Since the habit of sheep farmers in the creations in consortium with cattle in many often by the advantages that the larger animals offer in relation to the management of surplus forage grasses and and lowering grass, are nonexistent work which examine the frequency of toxoplasmosis in sheep herds grazing associated with cattle, and has been held to its relation with the presence of oocysts of *T. gondii* in the environment, especially in samples of soil and water. Thus, current literature provides a good bibliographical material resulting from scientific research that analyzed the frequency of anti-*T.gondii* antibodies in sheep, but is lacking according to the aspects mentioned above, precisely the purpose of this pioneering research in Brazil.

Nevertheless, for the frequency of anti-*T. gondii* of antibodies in sheep presented in Table 1, one can infer that the findings of this study are consistent with other studies conducted in São Paulo by other authors. Figlioulo *et al* (2004) identified 34.7% (207/597) of sheep serum reagents; Da Silva *et al* (2002) to research in 100 sheep serum for the toxoplasmosis by RIFI, randomly received for diagnosis of other disease, reported 23% seropositivity.

The frequency of sheep reagents to *T. gondii* in four properties in the region of Sorocaba, SP, proved to be inferior when compared with ovines in other regions of Brazil. In the state of Paraná, Ogawa *et al* (2003) reported the frequency of 54.6% (185/339) of anti-*T. gondii* antibodies in sheep using the RIFI; Garcia *et al* (1999), in a study of 228 sheep from 11 farms in the municipality of Jaguapitã determined a seroprevalence of 51.8% from a total of 228 sheep and Romanelli *et al* (2007) estimated the prevalence of 51.47% (157/305) of sheep positive to anti-*T. gondii* antibodies by RIFI.

Da Silva *et al* ^a registered 67.6% (173 sera) in sheep in the Zona of Mata and 32.4% of sheep serum reagents by RIFI in the region of Agreste. Was concluded, therefore, that in this case to the high prevalence of toxoplasmosis, moisture and vegetation type contributed to the formation of a favorable microenvironment for the maintenance of oocysts in the soil.

T. gondii can be transmitted vertically by tachyzoites that are passed to the fetus through the placenta (Tenter *et al.* 2000). At first contact with the *T. gondii*, sheep and goats in pregnancy can establish a placental and fetal infection (Buxton 1998), while that in the infected ewes before of the pregnant, do not exhibit reproductive losses (Beverley and Watson 1971).

In Minas Gerais, Carneiro (2006) analyzed sampling serologic from 711 sheep originating from the region Center-West and South of state found a seroprevalence of 43.2% using RIFI and 31.2% by ELISA. In the state of Rio Grande do Norte, Clementino *et al* (2007), using only the ELISA, determined seroprevalence similar in 29.41% in analysis of 102 sheep.

In present research, in relation to cattle grazing associated with sheep, were found values higher than those reported in other regions of the country, observing 94.12% reagents.

In the state of Parana, Marana *et al* (1995) identified 48.51% of dairy cattle as reagents to RIFI, while Garcia *et al* (1999) estimated the seropositivity in 25.8% among the cattle originated in the municipality of Jaguapitã. Similar results were found by Daguer *et al* (2004), while investigating the seroprevalence in cattle from slaughterhouses microregion of Pato Branco, found 41.4% (144/348) of reagents by RIFI.

Results less of frequency of anti-*T. gondii* antibodies in cattle were also found by Spagnol *et al* (2009) to study 600 serum samples from cattle originating from slaughterhouses, in Bahia of state. The authors determined 11.83% (71/600) for the total number of reacting animals, being that in the municipality of Ilheus, the percentage of reagents was 19.1% (37/192), 9.8% (21/214) in Itabuna and 6.7% (13/21) in Jequié.

In this research, so generally, RIFI titers of antibodies ranged from 1:64 to 1:1024 in both herds A and B of ovines farm exclusive. Both A and B herds, the titers were gradually distributed with greater frequency until the title of 1:256 to 1:1024 and decreases up to 1:1024. On the other hand, the eco-epidemiological study of the properties revealed that the property A showed the effect of intense promiscuity with cats on the a positivity of the herd. The animals (herd A) reacted with anti-*T. gondii* titers relatively high, which ranged from 1:64 to 1:512 (Table 2; Figure 1).

Still, in present research, in the farms of the sheep farming intercropping with cattle, 27% of sheep (herd C), 26% of sheep (herd D), 100% of cattle (herd C) and 93% of cattle (herd D) had *T. gondii* antibodies by RIFI (Table 1). The interval of frequency obtained for the herds ranged from 1:64 to 1:1024 and the frequencies of higher titers of anti-*T. gondii* in herds (C and D) of sheep in consortium with cattle ranged of 67% (1:64, C cattle), 50% (1:128, sheep C) and to 43% (1:512, sheep D), being only 11 % (D sheep) with titers of 1:1024 (Figure 2). Similar results to of this research, were obtained by Garcia *et al* (1999) 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 by Da Silva *et al* (2001) with titers ranging from 1:16 to 1:1024 sheep herd.

In present research, since was not performed in the region studied the serological analysis of cattle herds not consortium with sheep, there is no way to infer the possible interrelationship among species. However, two important risk factors investigated, could determine the presence of *T. gondii*, stock breeding conditions, the four flocks: the constant presence of domestic feline, cat, and free access to surface water, originating from soil leachate for troughs, animals and herds.

In present study, diagnosis by RIFI for reagents *T. gondii* in the sampling of mice in an experimental study through of inoculation of suspension of surface water for the clustering of water sampling, showed higher overall average 57.69% (15/26) of mice inoculated intraperitoneal (IP) versus 43.59% (17/39) of mice inoculated oral (VO) route, RIFI reagents. Only among the set of properties of group A (VO - 60 dpi, 0%) differed ($P<0.05$) mean of 11.11% (30 dpi - VO), in relation to route IP in others properties (Table 2), which led to believe that the RIFI can be used on equal conditions for the two routes of inoculation.

In this research, 53.85% of the titers of the mices bioassay with a water sampling, ranged from 1:16 to 1:256, and 57.69% ranged from 16 to 128 in mices inoculated via IP versus 38.46 % of titers ranging from 16 to 32 and 28.20% of the titers of 1:16 in mices inoculated via VO (Table 2).

In this study, the proportion of sheep on the property reagent A, was significantly higher than in the other ($P<0.001$). The promiscuity of the domestic cat, the definitive host of *T. gondii*, with the sheep herd of the property 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.

In the properties studied in this research, the eco-epidemiology of toxoplasmosis was strongly influenced by living together with domestic felines (cats). Moreover, there was no history of origin of the cattle. The grazing intercropping of sheep with cattle in a way, difficult the interpretation of the high frequencies found in bovine serum reagents, especially in relation to the role of the transmitters within species or between species. Apparently, 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, in the properties in the group A. These felines, has by habit after defecation, cover the faeces with soil in the various areas for grazing animals (Table 2).

The bioassay in mice is the standard test for detecting infection in tissues (Homan *et al.* 2000), due to its high sensitivity and specificity (Garcia *et al.* 2006) however, is laborious, costly, time consuming and offers risks to the operator (Esteban-Redondo *et al.* 1999).

In this research, though the mouse bioassay which detects the samples of surface water, water drinker of four farms had oocysts (Tables 2 and 3), was not observed equivalence between the frequency of animals serum reagents between them. Of these observation, it could be inferred that the viability of oocysts in the environment would be compromised, limiting the dissemination of the agent between animals; which in a sense, too emphasizes the important role of disseminator feline, created in property-A.

Regarding the capacity of IP and VO routes of inoculation of samples of surface water to produce infection in mice bioassay, in this research, could be inferred at least one hypothesis: the infective dose of

oocysts present samples of the property-A, would be able to produce infection in mice bioassay, regardless of inoculation IP or VO route. Fact reverse, was observed for samples of surface water from this property, where only the IP route was significantly more infectious than VO. Property-A, for its environmental conditions and sloping topography, besides allowing the sheep had access to water contaminated by *T. gondii* oocysts, this condition was 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 property (Tables 2 and 3).

The sampling to bioassay, showed the higher titers that ranged from 1:16 to 1:256 for 60% (30 dpi) and 50% (60 dpi) of mice inoculated via IP versus titers ranging from 1:16 to 1 : 32 to 43.33% (30 dpi) and 40% (60 dpi) from mice inoculated via VO (Table 2).

Domestic cats, play an important role in the epidemiology of toxoplasmosis (Dubey *et al.* 1986), since, contaminating water, soil and pasture, with oocysts eliminated by their faeces (Plant *et al.* 1974; Coutinho *et al.* 1982; Van Der Puije *et al.* 2000) can therefore be said that the oocysts eliminated, resulting in environmental contamination (Dubey 2004). Infection rates in cats, are usually determined by the rates of infection in populations of birds and rodents, which serve as food source (Dubey 2004). 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 (Dubey and Frenkel 1972).

The congenital transmission between sheep and lambs, often constitute a cause of abortion (Freyre *et al.* 1999; Duncanson *et al.* 2001; Tenter *et al.* 2000) and death during fetal development, birth and development of lambs rarely occur (Duncanson *et al.* 2001), there is still the possibility of occurrence of mummified fetuses (Buxton *et al.* 1998).

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 (Lindsay *et al.* 2001). The oocysts of *T. gondii* may persist in the environment for a long time (Villena *et al.* 2004).

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 (Tenter *et al.* 2000).

Diagnosis by polymerase chain reaction (PCR) in lung, liver and brain tissues of mice inoculated with suspension of surface water for the clustering of sampling, in this research, revealed a more effective with a mean significantly ($P < 0.05$) greater than 57.69% (15/26) of responses in the intraperitoneal (IP) for the PCR reagents versus only 2.56% (1 / 39) of mice inoculated orally (VO). Among the three organs analyzed by PCR lung, liver and brain, 57.69% (15/26) samples of mice were positive for the lung of animals inoculated IP and only 2.50% (1 / 39) of the lungs mice inoculated VO the PCR were positive, showing that the fabric of choice to study the PCR test, should be the lung (Table 3).

The polymerase chain reaction (PCR) is widely used for detecting *T. gondii* in amniotic fluid, blood and body tissue (Montoya *et al.* 1976), as well as in water (Isaac-Renton *et al.* 1998; Costa-Silva *et al.* 2010; Montoya *et al.* 1976; Dubey *et al.* 2004).

To Kourenti *et al.* (2003), 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.* (2004), 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 mices bioassay was negative, mainly with relation the application of sucrose flotation.

Sroka *et al.* (2006), in Loblin region (east of Poland) examined 114 samples water, of which the presence of DNA of *T. gondii* 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, using It is the commercial Genomic Mini Kit, A & A Biotecnology, Gdynia, Poland, and to amplify the DNA with the PCR DNA-GDNSK II Kit.

Similar results to this research were presented by Dubey *et al.* (2004) through the bioassay test 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.

Da Silva *et al.* (2001) 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), 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.

Although the PCR technique may be successfully employed in various diagnostic procedures due to its high sensitivity and specificity, in this research, it was not possible to detect the presence of DNA from oocysts in surface water and drinking fountain and samples of soil in four properties 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 (Sroka *et al.* 2006).

For the analysis of Table 3, was noted among the organs of mice bioassay analyzed by PCR, that the lung was the organ of choice in detecting DNA from *T. gondii*, independently of inoculation route (IP or VO) adopted.

Conclusions:

Summarizing the results of this research allowed concluded that all the properties studied, showed frequencies of serum-reagents animals the toxoplasmosis, relatively higher. Aggravating fact, the possibility of dissemination of protozoonosis, via contamination of water and soil, was the constatation of the promiscuity of the herd-A with the definitive host, the domestic felines; The eco-epidemiological study through of sampling for different forms of offering or availability of water to animals in different herds showed significant influence of the presence of domestic felids (cats), on the frequencies of animals reagents, the protozoonosis *T. gondii* detected by RIFI, PCR and to bioassay; In the eco-epidemiological study, water sampling and its different forms of availability of different herds of animals, can be inferred the influence definitive stray cats or domestic, in the properties studied; For analysis of water properties studied, the bioassay for different routes of inoculation (IP or VO), was more efficient than the PCR performed in supernatants water samples; The lungs of mice, proved to be the organ of choice after studying the bioassay and detection of *T. gondii* PCR; All samples of water 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 techniques.

ACKNOWLEDGMENTS

The authors would like to thank Profa. Dra. Luciana Regina Meirelles of Institute of Tropical Medicine of University of São Paulo - SP, by collaborate unconditionally and to provide of *T. gondii* strains.

Financial Support:

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) supported the present research (process number 2008/55384-0) and scholarship for technical capacitation (process number 2009/02033-8).

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