Serologic and molecular diagnostic and bioassay in mice for detection of *Toxoplasma gondii* in free ranges chickens from Pantanal of Mato Grosso do Sul[1]

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ABSTRACT.- Holsback L., Pena H.F.J., Ragozo A., Lopes E.G., Gennari S.M. & Soares R.M. 2012. **Serologic and molecular diagnostic and bioassay in mice for detection of *Toxoplasma gondii* in free range chickens from Pantanal of Mato Grosso do Sul.** *Pesquisa Veterinária Brasileira 32(8):721-726.* Setor de Veterinária e Produção Animal, Universidade Estadual do Norte do Paraná, Campus Luiz Meneghel, Rodovia BR 369 Km 54, Bandeirantes, PR 86360-000, Brazil. E-mail: lhsfertonani@uenp.edu.br

The aim of this study was to investigate the occurrence of *Toxoplasma gondii* and compare the results obtained in the Modified Agglutination Test (MAT), Polimerase Chain Reaction (PCR) and bioassay in mice. In order to accomplish this, 40 free-range chickens from eight farms in neighboring areas to the Pantanal in Nhecolândia, Mato Grosso do Sul, were euthanized and blood samples, brain and heart were collected. The occurrence of anti-*T. gondii* antibodies found in chickens was 67.5% (27 samples), considering as a cutoff point the dilution 1:5. Among the samples analyzed, 7 (25.9%) were positive in the dilution 1:5, 3 (11.1%) in 1:10, 2 (7.4%) in 1:20, 3 (11.1%) in 1:320, 1 (3.7%) in 1:640, 3 (11.1%) in 1:1280, 2 (7.4%) in 1:2560, 4 (14.8%) in 1:5120 and 2 (7.4%) in 1:10.240. From the mixture of tissue samples (brain and heart) from the chickens analyzed, 16 (40%) presented electrophoretic bands compatible with *T. gondii* by PCR (gene B1). In the comparison of techniques, 59.26% positivity in PCR was revealed among animals that were seropositive in MAT (cutoff 1:5). From 141 inoculated mice, six (4.44%) died of acute toxoplasmosis between 15 and 23 days after inoculation. Surviving mice were sacrificed at 74 days after inoculation, and a total of 28 cysts were found in the brains of 10 distinct groups. From the seropositive hens, 27 bioassays were performed and 11 (40.7%) isolates were obtained. A greater number of isolations happened in mice that were inoculated with tissues from chickens that had high titers for anti-*T. gondii* antibodies. Chronic infection in mice was observed in nine groups (33.3%) from five different properties. Among the surviving mice, 25.6% were positive for *T. gondii* in MAT (1:25). From mice positive in PCR, 87.5% were also positive in MAT. Among the PCR-negative mice, 5.2% were positive for *T. gondii* in MAT. It can be concluded through this study that the occurrence of infecton by *T. gondii* in the rural properties studied was high, that PCR directed to gene B1 does not confirm the viability of the parasite, but it can be used as a screening method for the selection of chickens infected by *T. gondii*, that the animals with titer greater than 10 must be prioritized for the selection of animals for bioassay, since for them, the chances of isolating the parasite are greater and that seroconversion in experimentally infected mice is not a good indicator for isolating the agent.

INDEX TERMS: Toxoplasmosis, bioassay in mice, free-range chicken, parasite disease, *Toxoplasma gondii*, comparison of diagnosis techniques.

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RESUMO.- [**Diagnóstico sorológico e molecular e bioensaio em camundongo para detecção de *Toxoplasma gondii* em galinhas de criação livre do Pantanal do Mato Grosso do Sul.**] Os objetivos deste estudo foram investigar a ocorrência de *Toxoplasma gondii* e comparar os resultados obtidos no Teste de Aglutinação Modificada (MAT), Reação em Cadeia pela Polimerase (PCR) e o bioensaio em camundongos. Para tanto, 40 galinhas de criação livre de oito fazendas em áreas limítrofes ao Pantanal da Nhecolândia, Mato Grosso do Sul, foram eutanasiadas e amostras de sangue, o cérebro e o coração foram coletados. A frequência de anticorpos anti-*T. gondii* encontrada nas galinhas foi de 67,5% (27 amostras), considerando como ponto de corte a diluição 1:5. Entre as amostras analisadas, 7 (25,9%) foram positivas na diluição 1:5, 3 (11,1%) em 1:10, 2 (7,4%) em 1:20, 3 (11,1%) em 1:320, 1 (3,7%) em 1:640, 3 (11,1%) em 1:1.280, 2 (7,4%) em 1:2.560, 4 (14,8%) em 1:5.120 e 2 (7,4%) em 1:10.240. A partir da mistura de amostras de tecidos (cérebro e coração) das galinhas analisadas, 16 (40%) apresentaram bandas eletroforéticas compatíveis com *T. gondii* por PCR (gene B1). Na comparação das técnicas, revelou-se 59,26% de positividade na PCR entre os animais soropositivos no MAT (ponto de corte 1:5). Dos 141 camundongos inoculados, seis (4,44%) morreram de toxoplasmose aguda entre 15 e 23 dias após a inoculação. Os camundongos que sobreviveram foram sacrificados 74 dias após a inoculação, sendo encontrados 28 cistos nos cérebros de 10 grupos distintos. Das galinhas soropositivas, foram realizados 27 bioensaios e obtidos 11 (40,7%) isolados. Um maior número de isolamentos ocorreu em camundongos que foram inoculados com tecidos de galinhas que tinham altos títulos de anticorpos anti-*T. gondii*. Infecção crônica em camundongos foi observada em nove grupos (33,3%) de cinco propriedades diferentes. Entre os camundongos que sobreviveram, 25,6% foram positivos para *T. gondii* no MAT (1:25). Dos camundongos positivos na PCR, 87,5% também foram positivos no MAT. Já entre os camundongos PCR negativos 5,2% foram positivos para *T. gondii* no MAT. Concluiu-se através deste estudo que a ocorrência de infecção pelo *T. gondii* nas propriedades rurais estudadas foi alta, que a PCR direcionada ao gene B1, não confirma a viabilidade do parasita, porém pode ser utilizada como método de triagem para a seleção de galinhas infectadas por *T. gondii*, que os animais com título maior que 10 devem ser priorizados para a seleção de animais para bioensaio, pois para eles, as chances de isolamento do parasita são maiores e que a soroconversão em camundongos infectados experimentalmente não é um bom indicador de isolamento do agente.

TERMOS DE INDEXAÇÃO: Toxoplasmose, bioensaio em camundongo, galinhas de criação livre, doença parasitária, *Toxoplasma gondii,* comparação de técnicas diagnósticas.

INTRODUCTION

Potentially capable of infecting many mammals and birds (Smith & Reduck 2000), *Toxoplasma gondii* is known to cause congenital disease and abortion in humans and domestic animals (Dubey & Beattie 1988, Remington & Desmonts 1990).

In Brazil, Vergara et al. (1985) showed that 70% of the human population has been exposure at some stage of life. Among several serological surveys already carried out have been identified in various regions Brazilian prevalence as 73.9, 74.7, 51.6, 78.7, 50.3 and 69% in region the Amazon, city of Recife, region the Xingu from state of Mato Grosso, Rio de Janeiro, Minas Gerais and São Paulo states, respectively (Araújo 1970, Baruzzi 1970, Coutinho et al. 1981, Jamra & Guimarães 1981, Ferraroni & Lacaz 1982, Azevedo et al. 1983, Guimarães et al. 1993).

Chickens, turkeys, ducks and canaries can be infected by *Toxoplasma gondii*, which confirms the eurixenic character of this parasite that may infect animals belonging to different groups. Resistance to infection might be related to age of animals because younger animals are the most affected (Amaro Neto et al. 1995). The role of breeding chicken in industrial scale in toxoplasmatic transmission to humans is of low importance, since their breeding is fast and contact with felines is not allowed. However, it contrasts with the domestic breeding in low scale, where the birds live for years in the same ecosystem as felines (Araujo et al. 1989, Literak & Hejlicek 1993). In this case, free-range chicken are susceptible to infection by *T. gondii* through feed or water contaminated with oocysts.

For years, papers are published about seroprevalence of toxoplasmosis in chickens using various methods of immunodiagnosis. The prevalence of 2.8% indirect hemagglutination test in chickens for slaughter from Rio Grande do Sul, was showed by Araujo et al. (1989) and Barbosa (2007) detected antibodies anti- *T. gondii* by modified agglutination test (MAT) in one (0.33%) of 300 sera of broilers from Belem, Pará state, from samples from poultry slaughtered illegally .

The infection and the disease have been detected with greater sensitivity by molecular methods of diagnosis such as Polymerase Chain Reaction (PCR). However, the limitation of the technique is to not distinguish whether the amplified DNA is derived from viable parasites or fragments of the parasite (Holliman 1994). Because of this, the bioassay in susceptible animals reproduce the infection when there is viable parasites in animal tissues, reflecting the active presence of the same. The objectives of this study were to determine the occurrence antibodies anti-*T. gondii* in adult free ranges chickens from farms in the Pantanal region of Nhecolândia, Mato Grosso do Sul, and compare the performance of PCR with the performance of MAT for diagnosing *Toxoplasma* infection in chickens naturally infected and in experimentally infected mice by bioassay.

MATERIALS AND METHODS

The state of Mato Grosso do Sul is located in mid-western Brazil and occupies an area of 358,158 Km^2. The climate is tropical humid with rainy summers and dry winters. The chickens were evaluated on two Municipalities and eight farms: Aquidauana, Farm 1, 3, 4, 5, 6 and 7 (19°17'17.0''S, 55°14'07.4''W; 19°14'30.0''S, 55°17'47.0''W; 19°28'31.1''S, 55°16'28.3''W; 19°20'48.8''S, 55°15'37.5''W; 19°17'09.1''S, 55°12'41.5''W and 19°27'16.1''S, 55°15'50.5''W respectively) and Rio Verde do Mato Grosso, Farm 2 and 8 (19°11'30.8''S, 55°15'46.9''W and 19°15'42.9''S, 55°11'17.3''W respectively) (Fig.1).

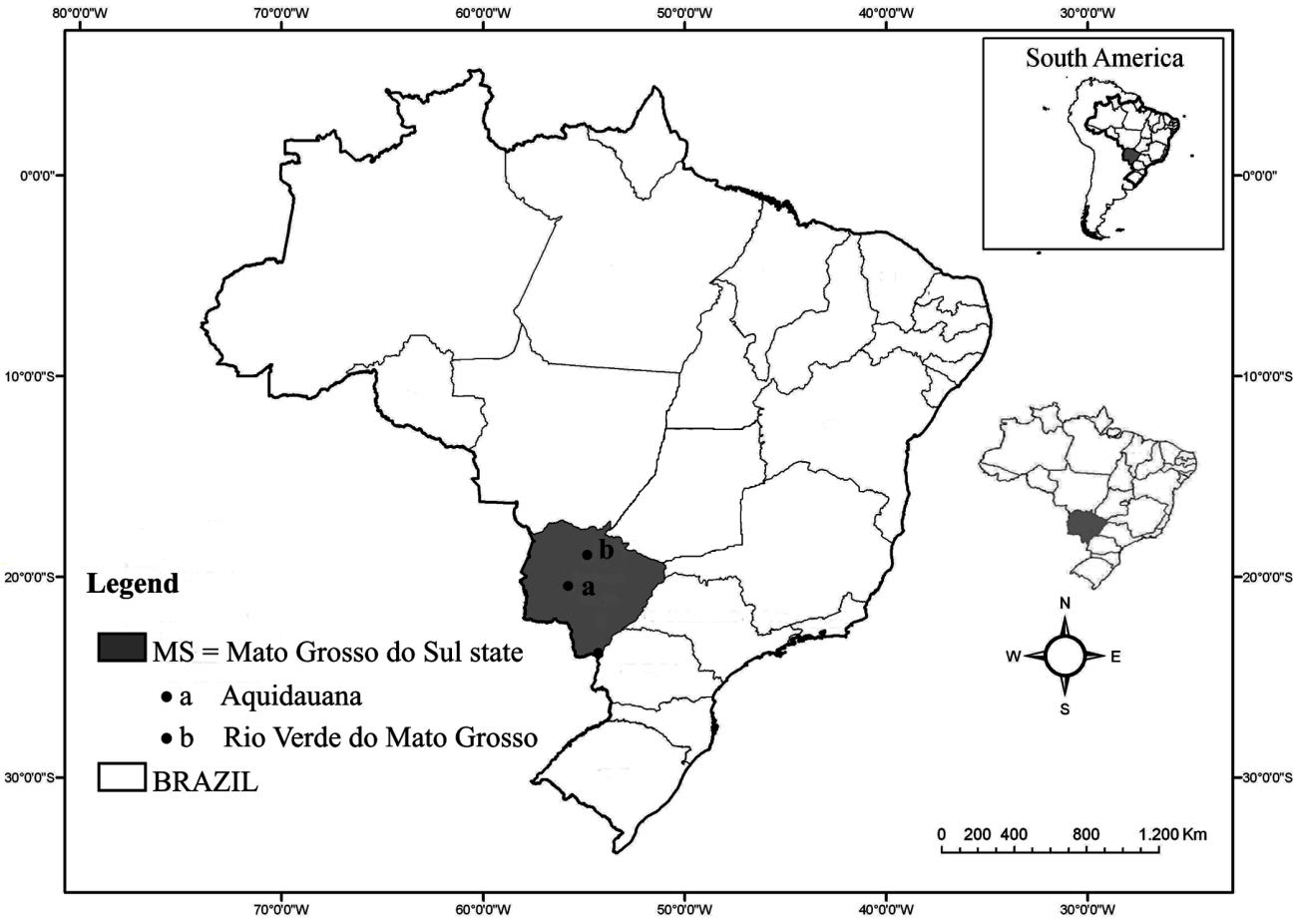

Fig.1. Map of Brazil with sources of sampled chickens.

A total of 40 free-range chickens from eight properties located in the Pantanal sub-region of Nhecolândia, in the state of Mato Grosso do Sul were used in this study. The approximate age of the chickens was 2 to 6 years, with males being the older animals. Regarding sex, 23 chickens were euthanized and 17 roosters in accordance with the choice of owners.

The chickens were killed by cervical dislocation and blood collected directly by incision of the jugular vein. Samples of blood, heart and brain from each chicken were individually identified and kept on ice and taken to the laboratory of Parasitic Diseases of the VPS-FMVZ-USP, São Paulo, at most three days after euthanasia. The carcasses were left with the owners for incineration.

Serum samples were examined by modified agglutination test (MAT) (Dubey & Desmonts 1987). The antigen was kindly provided by Dr. J.P. Dubey Laboratory of Parasitic Diseases of Animals, Department of Agriculture of the United States.

For screening of animals, dilutions of sera were performed in series of 1:5 to 1:40. Chickens with titers greater than or equal to 5 were considered positive (Dubey et al. 2003a) and titers greater than or equal to 40 were diluted and tested again until you reach the title maximum of the reaction. It was used positive and negative controls previously known.

For the bioassay were used Balb-c female mice about 2 months old. Each group (27 in total, G1 to G27) was formed by three to six mice kept in the same box. After inoculation, the mice were observed once a day. All mice were euthanized 74 days after inoculation by cervical dislocation with prior sedation for blood collection and detection of *Toxoplasma gondii*. The serum was examined by the modified agglutination test (MAT) (Dubey & Desmonts 1987). All mice used in the bioassay were examined for *T. gondii* in the tissues, as previously described (Dubey & Beattie 1988). The mice whose tissues were found some stage of the parasite, detected by direct examination of brain fragment or molecular diagnosis, were considered infected from *T. gondii*. Isolation was considered positive when there was amplification of parasite DNA using PCR, regardless of the visualization of biological forms (tachyzoites or bradyzoites) in tissues of mice bioassay.

DNA extraction was based on protocols described by Ausubel et al. (1999). For to increase the sensitivity of the technique, DNA extraction was performed in triplicate. Samples of brain and heart of all chickens was analyzed by PCR using the primer pair for gene B1 (F-5 'GGA ACT GCA TCC GTT CAT GAG3' and R 5'-TCT TTA AAG CGT TCG TGG TC-3'). The same was done in the tissues of inoculated mice. In each PCR run, a positive control (RH sample), at least two negative controls (pure autoclaved water) and extraction control (TE – Tris-EDTA buffer) were inserted.

The comparison of proportions and the statistical concordance indexes (kappa coefficient calculation) were obtained through the program EPITABLE (EPI-INFO 6.0). The Chi-square test was performed with the aid of the program STATCALC (EPIINFO 6.0).

RESULTS AND DISCUSSION

The frequency of anti-*Toxoplasma gondii* found in chickens was 67.5% (27 samples) considering a cutoff dilution 1:5. Of positive samples, seven (25.9%) were positive at 1:5 dilution, three (11.1%) diluted 1:10, two (7.4%) diluted 1:20, three (11.1%) at 1:320 dilution, one (3.7%) diluted

1:640, three (11.1%) diluted 1:1,280, two (7.4%) diluted 1:2,560, four (14.8%) diluted 1:5,120 and two (7.4%) diluted 1:10,240. The relative frequency of animals positive for *T. gondii* of all farms valued between 40 and 100%. The results of this study showed a high frequency of animals positive for *T. gondii* by MAT, which agrees with other findings in surveys conducted in Brazil (Dubey et al. 2006a, 2007a, De Oliveira et al. 2009).

The highest frequency of seropositive chickens free range by the modified agglutination test (cutoff 1:5) was found in Illinois (100%), followed by Nicaragua (85.7%) (Dubey et al. 2007b,c). In Brazil, the frequency of seropositive chickens *T. gondii* by MAT (1:5) ranged from 39% (São Paulo) to 100% (Alagoas) (Dubey et al. 2002, De Oliveira et al. 2009).

Probably due to difficult access to the regions prone to flooding, such as the sub-region where the animals were evaluated in this study, the publications are rare, leading to impairments in comparison of data obtained in this study to others already made. These reports include studies on the prevalence of toxoplasmosis in pregnant women in the state (Figueiró-Filho et al. 2005), whose result was only 0.42%, seroprevalence studies in animals such as horses by immunofluorescence (Larangeira et al. 1985) with 32.8% of positive animals and pampas deer (*Ozotocerus bezoarticus*) in the region near the Nhecolândia, and found 12% seropositive animals through various serological techniques (Ferreira et al. 1997). In the last decade, Silva (2005) evaluated 15 horses from seven rural property subregions of the Pantanal Mato Grosso do Sul (including the sub-region Nhecolândia) and showed only 1.33% of animals positive by hemagglutination test, however, no animals were positive in the sub-region Nhecolândia.

In the present study 59.26% of seropositive chickens were positive in PCR (cutoff dilution 1:5) (Table 1). The fair agreement (kappa = 0.486) between tests (MAT and PCR) in chickens may be explained by several factors such as cross-reactions in the MAT, DNA amplification of *T. gondii* nonviable in tissue of chickens. Other explaination is localization of the parasite in skeletal muscles of chickens or other organs that were not used in the bioassay. Besides the possibility of amplifying the DNA of a microorganism dead (Holliman 1994), another limitation of PCR is the possibility of false-negative results when the parasite is not present in the material used. For this reason DNA extraction was performed in triplicate to increase the sensitivity of the technique. Of the 16 positive samples from chickens in the PCR, 25% were obtained from third sample extracted, demonstrating that this test failure can be repaired by repeating the extraction.

Table 1. Number of chickens positive and negative in the modified agglutination test (MAT, cutoff 1:5) and Polymerase Chain Reaction (PCR)

MAT	PCR		Total
	Positive	Negative	
Positive	16	11	27
Negative	0	13	13
Total	16	24	40

X^2 = 12.84; P<0.001. *Kappa* = 0.486; regular agreement.

When considering the PCR as "gold standard" and analyzing the diagnostic sensitivity and specificity of MAT, it was concluded that the sensitivity of diagnosis by MAT (cutoff 1:5) was 100% and specificity of only 54.2%. However, when considering an upper cutoff point (1:40), the specificity rises to 100%. This could be due to the presence of elementary pulses nonspecific (for example, cross--reaction with *Hammondia*). This hypothesis is supported by analyzing the statistical correlation between values of positive animals in the MAT (cutoff 1:40) and the animals positive on PCR, showing an excellent agreement between these diagnoses (Kappa = 0.947, p<0.0001).

Six mice (4.44%) died from acute toxoplasmosis between 15 and 23 days after inoculation confirmed by observation of tachyzoites in peritoneal fluid, lung and liver through optical microscopy and PCR. The antibodies title of anti-*T. gondii* in both chickens whose tissues were inoculated in these mice was of 5,120. From 129 surviving mice, *T. gondii* cysts were found in the brain of 28 mice in 10 different groups. Higher frequency of cysts (P<0.0001, X2=52.87) was observed in mice inoculated with tissues from chickens with high anti-*T. gondii* antibody titers, but not necessarily from those with highest titers. Correlating the frequencies of cysts in mice and the titers in chicken, a moderate positive correlation (r=0.302) could be observed between these two parameters. This means that, although a greater proportion of mice with cysts were found among the ones inoculated with chicken tissues whose tittering for anti-*T. gondii* antibodies were medium or high, this relation was not directly proportional (Table 2).

Table 2. *P* values resulting from comparison of the ratios of the number of cysts in the brains of mice inoculated with tissues from chickens of different titles of antibodies to *Toxoplasma gondii*

Titers	Antibodies titers anti-*T. gondii*							
	5	10	20	320	640	1,280	2,560	5,120
5	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-
320	**< 0.05**	0.081	0.258	-	-	-	-	-
640	**< 0.001**	**< 0.001**	**< 0.05**	**< 0.05**	-	-	-	-
1,280	**< 0.001**	**< 0.001**	**< 0.05**	0.065	0.260	-	-	-
2,560	**< 0.05**	0.098	0.284	0.680	< 0.05	0.098	-	-
5,120	**< 0.05**	0.059	0.206	0.908	0.064	0.399	0.844	-
10,240	**< 0.05**	< 0.05	0.088	0.655	0.134	0.751	0.741	0.999

P<0.05 (statistical difference).

Correlation between these parameters associated was cited by Ragozo (2007), obtaining 69.2% isolation (cysts found in brains) in mice inoculated with tissues from sheep with 3200 titer. However, Yai (2007) did not find differences between the titers from antibodies in capybaras and the percentage of isolates obtained in bioassay mice. Pena (2004) also found greater frequency of isolation in groups of mice inoculated with tissues from cats with high titers for anti-*T. gondii* antibodies.

Chronic infection was observed in nine groups (33.3%) of five different properties and obtained 32 isolates (22.7%) from PCR analysis of samples of brains of mice.

Thirty-three mice (25.6%) were positive for *T. gondii* in the MAT (1:25). Of these, 28 were PCR positive and 5 negative. The number of mice positive and negative MAT and PCR are shown in Table 3.

The four mice positive PCR and negative MAT, were from four different groups (G3, G5, G6 and G14). The titles of the chickens inoculated in these mice, ranged from 320 to 10,240. Besides the samples were positive by PCR were found two cysts in the brains of mice in G3 and G14. Five mice MAT positive and negative by PCR were also from five distinct groups (G2, G3, G6, G14 and G21) and the titles of chickens between 5 and 5120.

Isolates of *T. gondii* from MAT-negative mice may be due to absence of seroconversion or low diagnostic sensitivity (87.5%). The lack of seropositivity in infected mice may also have occurred by a prozone effect on MAT. The absence of seroconversion in mice with positive isolation of *T. gondii* was not cited in any previous work done like this. However, Pena (2004) by diluting the serum of mice positive in the MAT, to verify the maximum degree of anti-*T. gondii* antibody titers, found in up to 409,600. Although this author has conducted a study with cats, this suggests that mice produce large quantities of antibodies which could explain the occurrence of the prozone phenomenon in MAT. Pena (2004), Yai et al. (2009) and Ragozzo et al. (2008) found no cysts in the brains of mice were seronegative, moreover, these authors did not perform molecular analysis of tissue samples from these mice.

Seven of 28 mice with cysts in the brain were negative by PCR and also had four who did not react serologically with antigens of *T. gondii* in the modified agglutination test (MAT). These four animals were inoculated with tissues from chickens with high titers of anti-*T. gondii* (over 320). Increased frequency of mice infected in the MAT was found between the groups of chickens inoculated with organs whose evidence of anti-*T. gondii* were 640 (80%), 10,240 (77.8%) and 1,280 (73.3%) (Q2 = 64.24, p<0.0001) as shown in Table 4.

Despite high levels of antibodies to be associated with higher levels of the parasite in tissues, mainly in the brain (Aigner et al., 2010), probably was not obtained isolating in these mice because there were few bradyzoites in the aliquot used what may have led to not capture these cells in DNA extraction and subsequent amplification in PCR. Even if the parasite DNA was extracted from this suspension, the same may not have been captured in the sample pipetted. Another reason could be that the parasite found at a location other than the brain, since no analysis was performed from pool of skeletal muscles of these animals (chickens).

Table 3. Number of mice seropositive and seronegative at MAT and positive and negative in the PCR

MAT	PCR		Total
	Positive	Negative	
Positive	28	05	33
Negative	04	92	96
Total	32	97	129

X^2 = 85.7; P<0.001. *Kappa* = 0.815; excellent agreement.

Table 4. Frequencies of isolation and seropositivity for *Toxoplasma gondii* by MAT between total mice bioassays distributed between the various titles of the chickens

Antibodies titers anti-*T. gondii* (chickens)	Amount of mouse bioassays	Isolation in mice (%)	Positive mice (MAT) (%) 1:25
5	39	0 [a]	2.6 [a]
10	17	0 [a]	0 [a]
20	9	0 [a,b,e]	0 [a]
320	15	26.7 [b,d]	26.7 [a,b]
640	5	100 [c,f]	80 [b]
1,280	15	60 [d,f]	73.3 [b]
2,560	11	27.3 [b,d]	9.1 [c]
5,120	9	33.3 [b,d]	55.6 [b,c]
10,240	9	44.4 [e,d]	77.8 [b]

Different letters in the same column represent statistically significant differences in chi-square test (P<0.05).

CONCLUSIONS

The occurrence of infection by *Toxoplasma gondii* in the farms studied was high; the PCR targeting the B1 gene, does not confirm of the viability of the parasite, but can be used as a screening method for the selection of chickens infected for *T. gondii*.

Chickens with titers greater than 10 should be prioritized for screening of animals for bioassay, because the chances of isolation of the parasite from them are larger.

The results of this study show that seroconversion in experimentally infected mice is not a good indicator for isolation of the agent.

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