

VIRULENCE FOR BALB/C MICE AND ANTIGENIC DIVERSITY OF EIGHT *TOXOPLASMA GONDII* STRAINS ISOLATED FROM ANIMALS AND HUMANS IN BRAZIL

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Summary :

With the purpose of establishing alternative parameters to determine the virulence of *Toxoplasma gondii* strains, the antigenic diversity of eight strains of the parasite isolated in Brazil was evaluated. BALB/c mice were inoculated i.p. with 10^0 , 10^1 , 10^2 and 10^3 tachyzoites from each strain. The mortality and time to death of the animals showed that *T. gondii* strains may be divided in three groups: three strains resulted in 100% of mortality, 5-10 days post inoculation (DPI); three strains resulted in 100% of mortality, 7-19 DPI and brain cysts were observed in the mice which were inoculated; two strains resulted in 0% of mortality, 30 DPI. The analysis of the antigenic profile of different *T. gondii* strains through Western blotting, using rabbit antiserum to *T. gondii*, revealed that most antigens are similar to all strains. The mAb 4C3H4 recognized antigens only in the RH, N, AS28 and ME49 strains..

KEY WORDS : *Toxoplasma gondii*, strain, Brazil, virulence, Western blotting.

Résumé : VIRULENCE POUR LE SOURIS BALB/C ET DIVERSITÉ ANTIGÉNIQUE DE HUIT SOUCHES DE *TOXOPLASMA GONDII* ISOLÉES À PARTIR D'ANIMAUX ET D'HOMMES AU BRÉSIL

Dans le but d'établir des paramètres alternatifs pour déterminer la virulence de souches de *Toxoplasma gondii*, la diversité antigénique de huit souches du parasite isolées au Brésil a été évaluée. Les souris BALB/c ont été inoculées i.p. avec 10^0 , 10^1 , 10^2 et 10^3 tachyzoïtes de chaque souche. La mortalité des animaux a montré que les souches de *T. gondii* peuvent être réparties en trois groupes : trois souches ont résulté en 100 % de mortalité, 5-10 jours après l'inoculation (DPI); trois souches ont entraîné 100 % de mortalité, 7-19 DPI et des kystes ont été observés dans le cerveau des souris qui ont été inoculées; deux souches ont entraîné 0 % de mortalité, 30 DPI. L'analyse du profil antigénique de différentes souches de *T. gondii* en Western blotting utilisant un antisérum de lapin contre *T. gondii* a révélé que la plupart des antigènes sont semblables à toutes les souches. Seules quatre souches (RH, N, AS28 et ME49) ont été reconnues par l'anticorps mAb 4C3H4.

MOTS CLÉS : *Toxoplasma gondii*, souche, Brésil, virulence, Western blotting.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite protozoan that infects a great variety of vertebrate hosts throughout the world, including human beings (Dubey & Beattie, 1988).

The prevalence of infection by *T. gondii* in human beings is high, with estimates of chronic infection in adult individuals varying from 15 to 85 % depending on the geographical region (Dubey & Beattie, 1988). However, the infections are typically asymptomatic, being able to cause severe lesions in immunocompromised patients and in congenitally infected fetuses (Luft & Remington 1988; 1992).

Toxoplasma gondii is recognized as the only species of the genus. However, *T. gondii* strains vary in their virulence. *T. gondii* has been defined as virulent, avirulent or of intermediate virulence, depending on its morbidity and mortality in mice (Guo & Johnson, 1995; Howe & Sibley, 1995). The RH strain (Type I) and those strains which are genetically similar to, are always lethal to mice, irrespective of dose or the strain of the mouse. In contrast, avirulent strains (Type III) show an LD₁₀₀ greater or equal to 10^3 parasites and chronic infections are easily established in mice (Howe *et al.*, 1996). The strains with intermediate virulence (Type II) seem to be strains in transition between the virulent and avirulent phenotypes of the parasite (Literák *et al.*, 1998).

T. gondii strains have been categorized through studies of isoenzymes (Dardé *et al.*, 1992), Restriction Fragment Length Polymorphism (Cristina *et al.*, 1995; Howe & Sibley 1995; Literák *et al.*, 1998), RAPD-PCR (Guo *et al.*, 1997), antigenic analysis by Western blotting (Ware & Kasper, 1987; Weiss *et al.*, 1988; Appleford & Smith, 2000) and reactivity with monoclonal antibodies (Gross *et al.*, 1991; Bohne *et al.*, 1993).

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With the aim of characterizing eight *T. gondii* strains isolated in Brazil, we evaluated the virulence for BALB/c mice and antigenic diversity of tachyzoites of different strains through Western blotting with rabbit antiserum to *T. gondii* and with 4C3H4 monoclonal antibody (Elsaid *et al.*, 1999).

MATERIALS AND METHODS

TOXOPLASMA GONDII STRAINS

Eight *T. gondii* strains isolated in Brazil (São Paulo state and Minas Gerais state) were examined: AS28, BV, N, EGS, RAR, SAF, C4 and P. The RH and ME49 strains were also studied as they represent, respectively, virulent and avirulent strains of the parasite (Howe & Sibley, 1995). The origin, place and year of isolation of *T. gondii* strains analyzed in this study are presented in Table I.

The tachyzoites of *T. gondii* were obtained by inoculating from 250 to 500 cysts or 10^5 to 10^6 tachyzoites of different strains by intraperitoneal (i.p.) injection in Swiss mice. The peritoneum of these mice was washed two to eight days post inoculation (DPI) and the resulting material was filtered through polycarbonate membrane of 3 μm (Millipore). The parasites were counted and diluted for appropriate concentrations (10^3 , 10^2 , 10^1 and 10^0 tachyzoites) in PBS pH 7.2.

Strain	Origin	Place/Year of isolation	References
AS28	Mice	São Paulo/1969	Deane <i>et al.</i> , 1971
BV	Goat	Minas Gerais/1975	Chiari <i>et al.</i> , 1985
N	Rabbit	São Paulo/1952	Nóbrega <i>et al.</i> , 1952
EGS	Human (CT)	Minas Gerais/1998	Castro, 1999
RAR	Human (CT)	Minas Gerais/1998	Castro, 1999
SAF	Human (CT)	Minas Gerais/1998	Castro, 1999
C4	Dog	São Paulo/1972	Jamra & Vieira, 1991
P	Dog	São Paulo/1978	Jamra & Vieira, 1991
RH	Human	EUA/1939	Sabin, 1941
ME49	Sheep	EUA/1965	Dardé, 1992

CT = Congenital toxoplasmosis

Table I. – Origin, place and year of isolation of *Toxoplasma gondii* strains analysed in this study.

DETERMINATION OF VIRULENCE OF *T. GONDII* STRAINS IN MICE

Female BALB/c mice, from six to eight weeks of age, were used for experimental inoculations. For each strain, five animals were inoculated i.p. with each one of the concentrations of tachyzoites and the mortality and time to death were observed for a period of 30 days. Five normal animals inoculated i.p. with PBS pH 7.2 were maintained as negative control. The mice were kept under conventional conditions and fed with pelleted food.

On the 30th day after inoculation, the surviving mice were bled by the retro-orbital plexus and the sera were tested by indirect fluorescent antibody test (IFAT), performed in accordance with the technique described by Camargo (1964). The mice which did not seroconvert were excluded from the experiment. All of the surviving mice were sacrificed by cervical dislocation for searching tissue cysts in the brain.

PREPARATION OF *T. GONDII* ANTIGEN FOR WESTERN BLOTTING

T. gondii tachyzoites were withdrawn from Swiss mice infected as described above. The tachyzoites were washed three times with PBS pH 7.2 by centrifugation at 1,500 g, for 15 minutes and filtered in polycarbonate membrane of 3 μm (Millipore). Aliquots of 10^4 tachyzoites were stocked at -20°C until the moment of use. Cells obtained from peritoneal cavities of Swiss mice not infected with *T. gondii* were purified by using the same technique described above. The RAR strain was not studied by Western blotting.

SDS-PAGE AND WESTERN BLOTTING

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransference of proteins to the nitrocellulose membrane of 0.45 μm porosity were performed in accordance with what was previously described (Vitor *et al.*, 1999). After transference, the membranes were blocked with skimmed milk at 10 % in PBS-Tween 20 0.05 % for two hours. Afterwards, the membranes were incubated with rabbit antiserum to *T. gondii*, immunized with dead tachyzoites of N strain, diluted 1:50 in PBS containing bovine serum albumin (BSA) at 3 %, for one hour under agitation at room temperature or with the monoclonal antibody, 4C3H4, anti-P32, developed against tachyzoites of the N strain (Elsaid *et al.*, 1999). After three washings in PBS-Tween 20 0.05 %, the membranes were incubated with anti-immunoglobulin G (IgG) of rabbit conjugated with peroxidase or anti-IgG of mice conjugated with peroxidase (SIGMA) in 1:5000 dilution in PBS pH 7.2 for 1h, at room temperature. Proteins were shown by developing membranes by using 4-chloro-1-naphthol as substrate.

STATISTICAL ANALYSIS

The differences observed between the mean day of mice death inoculated with different strains of *T. gondii* and among the means of the number of brain cysts in surviving mice after 30 days of infection were analyzed by Student's *t* test, using the significance level of 95% (Armitage & Berry, 1994). The correlation between the number of *T. gondii* tachyzoites inoculated and the number of brain cysts in the mice was analyzed by the Linear Regression (Armitage & Berry, 1994).

RESULTS

VIRULENCE OF *TOXOPLASMA GONDII* STRAINS IN BALB/C MICE

Table II presents the virulence comparison for BALB/c mice infected i.p. with different inocula of tachyzoites of *T. gondii* strains analyzed in this study.

Infections with RH, AS28, BV, and N strains resulted in 100 % of mortality of mice, with the death of animals occurring 5-10 DPI, with the exception of the inoculum of 10¹ tachyzoites of the RH strain, in which one mouse (20 %) survived 30 DPI. Nevertheless, this mouse presented negative serology by IFAT and negative brain examination, suggesting that the animal was not infected. Brain cysts were not observed in any of the mice inoculated with these strains.

The infections with the EGS, RAR, and SAF strains resulted in 100 % of mortality from seven to 19 DPI. For the inoculum of 10⁰ tachyzoites of the RAR strain, only one mouse (20 %) died 18 DPI. Meanwhile, the surviving mice presented negative serology for *T. gondii* by IFAT, without brain cysts.

Brain cysts were observed in mice inoculated with EGS, RAR and SAF strains. Time to death was significantly longer than that observed for mice infected with different inocula of tachyzoites of the RH strain (P < 0.05), except for the inoculum of 10³ tachyzoites.

The ME49, C4 and P strains were not virulent for mice in the inocula effectuated. All animals survived after the 30-day-period of observation, with the exception of a mouse inoculated with 10² tachyzoites of the P strain, which died 19 DPI. The brain of this animal was positive for tissue cysts.

BRAIN CYSTS IN BALB/C MICE INOCULATED WITH ME49, C4 AND P STRAINS OF *TOXOPLASMA GONDII*

Brain cysts were found in all mice inoculated with C4 and P strains, surviving 30 DPI and which presented positive serology for *T. gondii*. Brain cysts were found in 2, 4, 1 and 0 mice inoculated, respectively, with 10³, 10², 10¹ and 10⁰ tachyzoites of the ME49 strain. All of them were positive by IFAT.

As presented in Table III, the number of brain cysts in mice infected with C4 and P strain was significantly different than that found in mice infected with the ME49 strain (P < 0.05). The C4 strain formed a greater number of cysts than the P strain, in all of the tested

Strain	Number of tachyzoites inoculated i.p.	Number of surviving* / inoculated mice	Mean number of brain cysts ± SD
ME49	10 ⁰	4/5	-
	10 ¹	5/5	20 ± 44.7
	10 ²	5/5	80 ± 44.7
	10 ³	5/5	40 ± 54.8
P	10 ⁰	2/5	350 ± 70.71
	10 ¹	5/5	380 ± 258.8
	10 ²	4/5	600 ± 141.4
	10 ³	5/5	1,000 ± 254.9
C4	10 ⁰	4/5	500 ± 230.9
	10 ¹	5/5	1,960 ± 1,608.7
	10 ²	5/5	2,040 ± 1,556.6
	10 ³	5/5	2,000 ± 902.8

* mice with positive IFAT

Table III – Number of brain cysts in BALB/c mice 30 days post inoculation with tachyzoites of ME49, P and C4 strains of *Toxoplasma gondii*.

Strain	Number of tachyzoites inoculated i.p. (five mice each dilution)							
	10 ⁰		10 ¹		10 ²		10 ³	
	D/S*	d ⁺	D/S	d	D/S	d	D/S	d
RH	5/0	10,0	4/0	9,5	5/0	8,0	5/0	6,8
AS28	5/0	8,4	5/0	7,0	5/0	6,0	5/0	5,0
BV	5/0	8,8	5/0	8,0	5/0	7,2	5/0	6,0
N	5/0	8,8	5/0	8,0	5/0	7,2	5/0	6,8
EGS	5/0	19,2	5/0	13,2	5/0	9,4	5/0	7,0
RAR	1/0	18,0	5/0	16,2	5/0	13,0	5/0	10,2
SAF	5/0	18,8	5/0	16,4	5/0	10,8	5/0	8,4
ME49	0/4	-	0/5	-	0/5	-	0/5	-
C4	0/4	-	0/5	-	0/5	-	0/5	-
P	0/2	-	0/5	-	1/4	19,0	0/5	-

* D/S = dead/surviving mice (with positive IFAT)

+ d = mean day of death

Table II. – Virulence comparison for BALB/c mice inoculated by intraperitoneal injection with tachyzoites of different *Toxoplasma gondii* strains.

inocula. However, the number of cysts was significantly different only in mice inoculated with 10^3 tachyzoites ($P < 0.05$). In addition, there was a greater number of cysts in mice infected with greater inocula of tachyzoites. However, the correlation was significant only in animals infected with the P strain ($r = 0.96$).

ANTIGENIC CHARACTERIZATION OF *T. GONDII* STRAINS ISOLATED IN BRAZIL

Numerous antigens of similar molecular weight were identified by the rabbit antiserum to *T. gondii* in all strains: 80, 72-74, 67, 64, 56, 49, 44, 39, 36, 32 and 21 kDa (Fig. 1). There was no reactivity of antibodies with antigens of peritoneal cells of mice.

The mAb 4C3H4 reacted with antigens of 32, 21 and 15 kDa of the highly virulent N and RH strains, and with antigen of 26 kDa of the ME49 strain, not presenting reactivity with any of the other strains (Fig. 2). In order to verify if this mAb could recognize antigens of low expression in other strains, a greater number of tachyzoites (10^5 per lane) of RH, EGS, AS28 and N strains was used. As it can be observed in Fig. 3, the mAb reacted with antigens with molecular weight of 32, 21 and 15 kDa in RH, AS28 and N strains. There was no reactivity with tachyzoites of the EGS strain, confirming that the mAb 4C3H4 recognizes, preferentially antigens of *T. gondii* strains highly virulent for mice.

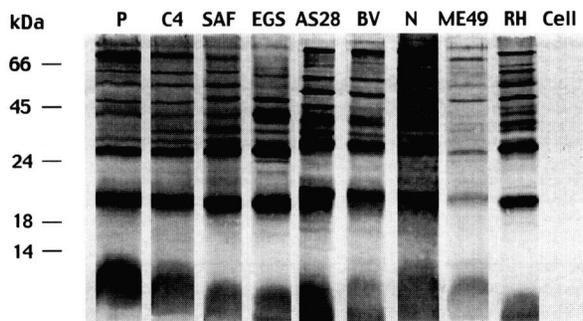


Fig. 1 – Western blotting analysis of antigens of P, C4, SAF, EGS, AS28, BV, N, ME49 and RH strains of *Toxoplasma gondii* reacted with rabbit antiserum to *T. gondii*. Equal numbers (10^4) of purified tachyzoites from each strain were subjected to 12,5 % polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. Molecular weight markers (in kilodaltons) are indicated on the left. Cell - cells obtained from peritoneal cavities of Swiss mice not infected with *T. gondii*.

DISCUSSION

In these experiments of mice inoculated with 10^0 to 10^3 tachyzoites of the AS28, BV, N, EGS, RAR, SAF, C4 and P strains of *T. gondii* isolated in Brazil, and of RH (virulent) and ME49 (avirulent) strains, reported in this study, it was observed that the *T. gondii* strains

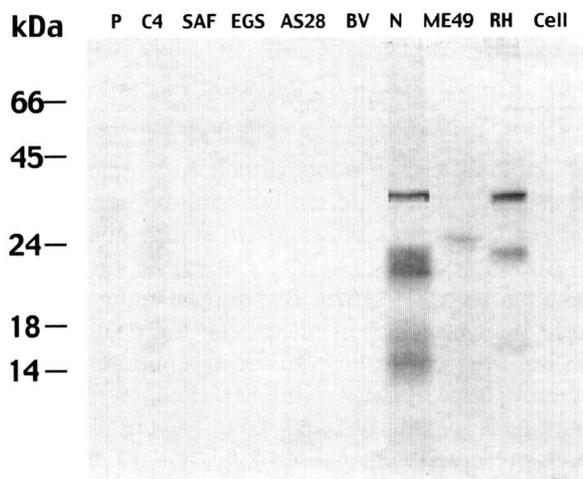


Fig. 2 – Western blotting analysis of antigens of P, C4, SAF, EGS, AS28, BV, N, ME49 and RH strains of *Toxoplasma gondii* reacted with mAb 4C3H4. Equal numbers (10^4) of purified tachyzoites from each strain were subjected to 12,5 % polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. Molecular weight markers (in kilodaltons) are indicated on the left. Cell - cells obtained from peritoneal cavities of Swiss mice not infected with *T. gondii*.

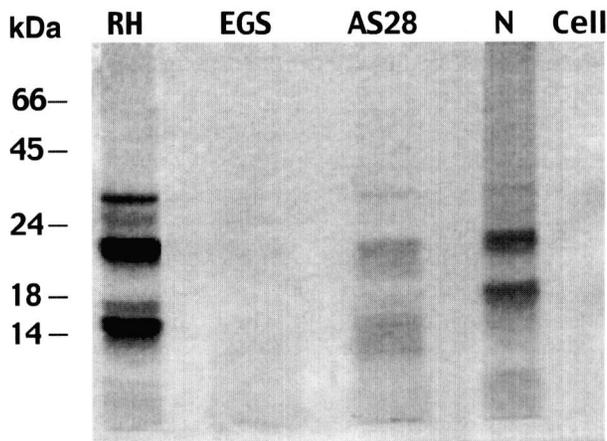


Fig. 3 – Western blotting analysis of antigens of RH, EGS, AS28 and N strains of *Toxoplasma gondii* reacted with mAb 4C3H4. Equal numbers (10^5) of purified tachyzoites from each strain were subjected to 12,5 % polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane. Molecular weight markers (in kilodaltons) are indicated on the left. Cell - cells obtained from peritoneal cavities of Swiss mice not infected with *T. gondii*.

analyzed may be divided in three groups, according to the virulence for BALB/c mice.

The AS28, BV and N strains presented high virulence, similar to that observed for the RH strain, with the mortality of all mice infected with the different number of tachyzoites. These strains have not produced brain cysts. However, as the animals died prematurely, the research of cysts may have had a false-negative result. In mice, tissue cysts are formed within three and four

days after parenteral inoculation with tachyzoites (Dubey & Beattie, 1988). Nevertheless, in the beginning of the infection, the visualization is made difficult by its small size. Howe & Sibley (1995) observed that highly virulent strains, as the RH strain, lost the ability to form tissue cysts or they form a very reduced number of cysts.

The EGS, RAR, and SAF strains, isolated from cases of human congenital toxoplasmosis formed the second group of strains observed in this study. Despite being virulent for mice, killing 100 % of animals, the presence of brain cysts was observed in mice which died after the infection and time to death was longer than that observed for the mice inoculated with the strains of the first group.

The C4 and P strains present an avirulent behavior, as the ME49 strain. The inoculation of tachyzoites of these strains led to the development of brain cysts without killing the mice, even with the inoculum of 10^3 tachyzoites. A mouse inoculated with 10^2 tachyzoites of the P strain died 19 DPI, exemplifying individual characteristics of response to the infections, even in populations of isogenic animals.

Within the strains analyzed in this study, the AS28 strain calls attention by the fact of having been primarily described causing an infection in mice tending to be chronic, developing a great number of brain cysts in the animals (Deane *et al.*, 1971). In our study, this strain showed to be highly virulent for mice, killing 100 % of the animals in less than 10 DPI, and the presence of brain cysts was not observed.

Jacobs & Melton (1954) showed that successive passage of tachyzoites from one strain in mice modifies the virulence of *T. gondii*. Nevertheless, we agree with Dubey & Frenkel (1973) who stated that the adaptation of a certain host differs among the several strains of *T. gondii*. For instance, the RH strain killed four of five mice infected in the first passage from 17 to 21 days soon after its isolation in 1939, but after only three intraperitoneal passages it started to kill all the mice inoculated from three to five days (Sabin, 1941). On the other hand, with M-7741 strain, even after 62 passages, the mice infected with 10^5 tachyzoites survived for more than nine days (Dubey & Frenkel, 1973).

Similarly, the C4 and P strains analyzed in this study maintain the same avirulent behavior since its isolation, while the BV and N strains, isolated from animals, and the EGS, RAR and SAF strains, isolated from human cases of congenital toxoplasmosis, killed 100 % of mice since the first passage after its isolation.

The number of brain cysts differed among the mice infected with the ME49, C4 and P strains of *T. gondii*. The number of brain cysts in mice infected with C4 and P strains was greater than the one found in mice infected with the ME49 strain. The C4 strain formed

more brain cysts than the P strain in mice infected with 10^3 tachyzoites. Suzuki *et al.* (1989) also observed differences in the number of cysts formed in the brain of CBA/Ca mice, infected with ME49 and DAG strains of *T. gondii*. The authors declared that the pathogenesis of encephalitis by *T. gondii* should be considered in the context of the strain of the parasite involved and its potential for a continuous activity during the acute infection in mice.

The analysis by Western blotting of different strains of *T. gondii* showed that antigens of similar molecular weight, varying from 21 to 80 kDa were identified in all strains. These data differ from the results described in the literature as, through the technique of Western blotting, it was verified that there is an antigenic diversity among different strains of *T. gondii* (Ware & Kasper, 1987; Weiss *et al.*, 1988; Appleford & Smith, 2000).

However, Cazabonne *et al.* (1994) performed a study of kinetics and characterization of excreted/secreted antigens from three strains of *T. gondii* of different virulence and observed that the antigens were similar for all the strains. These strains produced the same immunological response in mice. In our study, most antigens were similar to all strains analyzed, which probably occurred due to the fact that the polyclonal serum used is capable of recognizing antigens with the same molecular weight in the different *T. gondii* strains analyzed.

The reactivity comparison of *T. gondii* strains with mAb 4C3H4 (Elsaid *et al.*, 1999) revealed three antigens recognized only in the RH and N strains which are highly virulent for mice, and one antigen of 26 kDa, recognized only in the ME49 strain. The reactivity with more than one antigen suggests that the mAb 4C3H4 recognizes epitopes which are common in proteins of different molecular weight.

Using a greater number of parasites per lane, the mAb 4C3H4 also reacted with antigens of the AS28 strain, suggesting a low expression in this strain. Nevertheless, there was no reaction of the mAb with antigens of the EGS strain. These results suggest that the specific recognition of virulent strains by mAb 4C3H4 is related to the loss of the ability to make cyst. Studies of molecular biology may help to explain the function and relevance of these antigens, particularly referring to its role in the virulence of strains.

While analyzing the reactivity of mAb 5B10, developed against the RH strain, Gross *et al.* (1991) observed that this mAb detected an antigen of 23 kDa expressed by the virulent strains, but not by the strains with low virulence, isolated from clinical cases of toxoplasmosis in Europe. Bohne *et al.* (1993) differed virulent strains from avirulent ones of *T. gondii* by using the mAb TB6G5, developed against the NTE strain which reacted with eight avirulent strains for mice, but not

with virulent strains. Together with the monoclonal antibodies developed by these authors, the analysis of reactivity of *T. gondii* strains with mAb 4C3H4, used in this study, could be an additional parameter for the serological classification of *T. gondii* in virulent and avirulent strains for mice.

The results of the present study showed the occurrence of *T. gondii* strains of varied virulence in Brazil. Further studies using genetic approaches that are now available for *T. gondii* (Sibley *et al.*, 1999) will be of great value in establishing genetic relationship among these Brazilian *T. gondii* strains.

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