

CHARACTERIZATION OF *TOXOPLASMA GONDII* FROM DOMESTIC ANIMALS FROM MINAS GERAIS, BRAZIL

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Summary:

In an attempt to isolate and characterize *Toxoplasma gondii* from the State of Minas Gerais, Brazil, musculature samples from 72 pigs, 25 dogs, 28 free-range chickens and 50 chickens produced in industrialized farms were collected. Antibodies to *T. gondii* have not been detected in pigs, but were found in nine (40.9 %) out of 22 dogs, and in 15 (53.6 %) of 28 free range chickens. *T. gondii* was not isolated from pigs and industrialized chickens, but from eight dogs and 11 free range chickens. In order to determine *T. gondii* virulence, female BALB/c mice were inoculated with 10³, 10², 10¹ and 10⁰ tachyzoites of the 19 isolates. The strains RH (virulent) and ME49 (non-virulent) were used as references. Isolates were divided into three groups according to the virulence phenotype: five isolates were classified into virulent in mice, one into non-virulent and 13 into intermediate virulent. Nested-PCR of *T. gondii* SAG2 locus amplified DNA from 21 out of 22 DNA samples directly extracted from heart of free range chickens. These samples were genotyped through a PCR-RFLP assay. Seventeen (80.9 %) were classified into type I; one (4.8 %) into type III and three (14.3 %) into type I or II.

KEY WORDS : *Toxoplasma gondii*, strains, domestic animals, virulence, PCR-RFLP, Brazil.

Résumé : CARACTÉRISATION DE *TOXOPLASMA GONDII* CHEZ DES ANIMAUX DOMESTIQUES AU MINAS GERAIS, BRÉSIL

Pour isoler et caractériser des isolats de *Toxoplasma gondii* au Minas Gerais, Brésil, des échantillons de muscle de 72 porcs, 25 chiens, 28 poulets d'élevage fermier et de 50 poulets d'élevage industriel ont été examinés. Il n'a pas décelé d'anticorps anti *T. gondii* chez les porcs, par contre, ils ont été détectés dans le sérum de neuf chiens sur 22 (40,9 %) et de 15 poulets fermiers sur 28 (53,6 %). *T. gondii* n'a été isolé, ni chez les porcs, ni chez les poulets d'élevage industriel. Il a été isolé sur huit chiens et 11 poulets fermiers. Pour déterminer la virulence des isolats de *T. gondii*, des souris BALB/c ont été inoculées avec 10³, 10², 10¹ et 10⁰ tachyzoïtes de 19 isolats et avec des souches RH (virulente), outre avec la souche ME49 (non-virulente). Les isolats de *T. gondii* ont été classés en trois groupes selon le phénotype de virulence: cinq classifiés comme virulents pour des souris, un non-virulent, et treize de virulence intermédiaire. À l'aide du Nested-PCR – pour extraire des produits d'amplification du locus SAG2 de *T. gondii* –, on a identifié le parasite dans 21 échantillons d'ADN extraits à partir du cœur de 22 poulets fermiers. Ces échantillons ont été génotypés par PCR-RFLP; 17 (80,9 %) ont été classés de type I, un (4,8 %) de type III et trois (14,3 %) de type I ou II.

MOTS CLÉS : *Toxoplasma gondii*, isolat, animal domestique, virulence, PCR-RFLP, Brésil.

INTRODUCTION

Toxoplasmosis is a worldwide infection distributed among homoeothermic animals with a high prevalence in humans (15-85 % seropositivity) in different populations. Humans acquire toxoplasmosis through ingestion of tissue cysts in raw or undercooked meat or even through water and raw vegetables contaminated with oocysts from cat feces (Dubey & Beattie, 1988). Although the infection is usually benign, HIV immunodeficient patients and fetuses may develop severe neurological symptoms (Gellin & Soave, 1992). Serological studies have evidenced a high

infection rate in animals for human consumption, mainly pork, lamb and goat (Tenter *et al.*, 2000). According to these authors, pork is the main source of human *T. gondii* infection in the US. Literák & Hejlíček (1993) remarked that free range chickens may represent a risky source of contamination when manipulation of raw meat is performed with no hygiene measures. Moreover, such chickens keep direct ground contact, becoming a major instrument for determining the level of environmental contamination by *T. gondii* oocysts (Devada *et al.*, 1998). Dogs play a secondary role in *T. gondii* transmission in Brazil, once they are not a food source for man. On the other hand, Lindsay *et al.* (1997) have reported high risk rates of human contamination through direct contact with dogs.

Molecular analysis of *T. gondii* strains worldwide has shown that most strains fit into one of the three genotypes, designated as type I, II and III (Howe & Sibley, 1995). Howe *et al.* (1997) developed a typing system

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for *T. gondii* strains based on analysis of restriction fragments of the gene *SAG2*, amplified by nested-PCR, which allows a differentiation among strain genotypes of the three clonal lineages, previously described by Howe & Sibley (1995). In Brazil, this method was used for analyzing *T. gondii* isolates from São Paulo (Dubey *et al.*, 2002), Rio de Janeiro (Dubey *et al.*, 2003a) and Paraná (Dubey *et al.*, 2003b), which showed to be predominantly type I. Ferreira *et al.* (2006), using a multilocus PCR-RFLP, demonstrated that Brazilian strains present recombinant genotypes with typical alleles of the strains types I, II, and III in the majority of the loci. The RAPD-PCR and SSR-PCR analysis of these same strains placed them into two different clusters correlated with virulence for BALB/c mice (Ferreira *et al.*, 2004). The aim of the current study was to obtain *T. gondii* isolates directly from tissues of pigs, dogs and chickens in the State of Minas Gerais, Brazil, and to determine their virulence in BALB/c mice. We have also attempted to determine *T. gondii* genotype of the *SAG2* locus in chicken through PCR-RFLP, using readily obtained DNA from heart samples.

MATERIALS AND METHODS

PIGS

Muscle tissue from 72 pigs was bioassayed in outbred Swiss Webster mice. Out of these, a number of 37 heart samples were collected between September and October 1999 in a slaughterhouse in Santa Luzia, State of Minas Gerais (MG). The 35 other samples comprised 21 whole tongues and 14 whole hearts, obtained from slaughterhouse of Ribeirão das Neves, MG, in 2000. It was not possible to assure whether the pieces, heart and tongue came from the same animal. Swine sera could not be collected either.

DOGS

Diaphragms from 25 dogs with leishmaniasis were bioassayed. The samples were obtained from February to June 2000 and the animals were collected by the Center of Zoonosis Control in the city of Belo Horizonte, MG, and killed at the Universidade Federal de Minas Gerais (UFMG).

CHICKENS

Heart samples from 28 free range chickens in residences from the Minas Gerais State were collected in 2000 (six samples) and 2003 (22 samples) and bioassayed; 50 other heart samples from industrialized chicken farms acquired in a slaughterhouse in Belo Horizonte, MG, in 2001, were also evaluated. Muscle and serum from

each animal were transported to the Department of Parasitology, UFMG.

BIOASSAY IN MICE

Muscle samples from pigs, dogs and chickens were digested in accordance with Dubey *et al.* (1995), with no previous knowledge on the animal serological status. Samples of approximately 10 g (from pigs and dogs) and half of each chicken heart were grinded and submitted to digestion with pepsin at 37° C for 90 min. To each sample, phosphate buffer saline (PBS) pH 7.2 with penicillin (1,000 units) and streptomycin (100 µg/mL) were added. A volume of 0.5 ml from each sample was inoculated into non-infected Swiss mice, from the Animal House of UFMG, via intraperitoneal (i.p.). Dead mice or those with infection characteristics were assessed for tachyzoite or tissue cyst search. Surviving mice were killed at day 30 post-inoculation (p.i.), and a serum dilution of 1:16 to 1:64 from each mouse was tested for *T. gondii* antibodies by IFAT (Camargo, 1964). Brains were also analyzed in order to verify the presence of tissue cysts (Dubey & Beattie, 1988).

SEROLOGY

Sera from dogs and chickens were diluted fourfold from an initial dilution of 1:16 and assayed for *T. gondii* antibodies by IFAT. Tissue liquid, obtained according to Wingstrand *et al.*, (1997), from pig muscle samples at 1:40 dilution was assayed for *T. gondii* antibodies search through ELISA.

DETERMINATION OF *T. GONDII* ISOLATES VIRULENCE

T. gondii virulence in mice was determined in accordance with Ferreira *et al.* (2001). Successive dilutions from 10³ to 10⁰ tachyzoites were prepared in sterile PBS pH 7.2. 20 female BALB/c mice were inoculated with each *T. gondii* isolate. Mice were obtained from the Animal House of UFMG, aged between four and six weeks, and divided into four groups with five animals each. Each group was inoculated with different concentrations of tachyzoites: 10³, 10², 10¹ and 10⁰ tachyzoites/animal i.p. in a volume of 0.2 ml. Five control animals were inoculated with 0.2 ml of sterile PBS pH 7.2. The virulent strain RH and non-virulent ME49 were used as reference strains (Ferreira *et al.*, 2001). Mice mortality was daily observed throughout 30 days, when the survivors were tested for *T. gondii* antibodies (IFAT) and brain cysts.

GENETIC ANALYSIS OF CHICKEN ISOLATES

DNA extraction was performed according to Aspinall *et al.* (2002) in each heart sample of the 22 chickens collected in 2003. DNA extraction was not possible to be performed in heart samples obtained in 2000. The

presence of *T. gondii* was identified by means of the *SAG2* locus analysis through nested-PCR, as described by Howe *et al.* (1997). Products of *SAG2* amplification by nested-PCR were digested by the restriction enzymes *Sau3AI* and *HbaI* (Promega). Digested products were visualized in 5 % silver stained polyacrylamide gel (Santos *et al.*, 1993). As a reaction control, the strains RH (type I), ME49 (type II) and VEG (type III) (Fux *et al.*, 2003) were used.

RESULTS

T. gondii was not isolated from pigs. Antibodies against *T. gondii* were not found in their tissue liquids. Out of the 25 muscle samples from dogs, eight (32 %) isolates were classified into D1 to D8 (Table I). *T. gondii* was isolated from three dogs, from which sera was not possible to be obtained. Anti-*T. gondii* antibodies were found in nine of the 22 (40.9 %) dogs analyzed. *T. gondii* was isolated in five muscle samples from dogs with positive serology, but not in four animals with positive serology or in animals with negative serology (Table I).

Of the 28 muscle samples from free range chickens, 11 (39.3 %) *T. gondii* isolates were classified into CH1 to CH11 (Table II). Anti-*T. gondii* antibodies were found in 15 (53.6 %) chickens. The parasite was isolated in nine chicken hearts with *T. gondii* antibodies and two other chickens without specific antibodies (chickens 3 and 4). *T. gondii* was not isolated in six chickens with positive serology or in samples obtained from industrialized chicken farms in Belo Horizonte.

Results on virulence in infected BALB/c mice inoculated with different concentrations of tachyzoites from *T. gondii* isolates are showed in Table III. Virulence experiments were done in duplicate and were repro-

Dog n°	IFAT titer	Isolation/Isolate name	Month/Year of isolation
1	ND	Yes/D1	02/2000
2	ND	Yes/D2	04/2000
3	ND	Yes/D3	04/2000
4	1:1024	Yes/D4	06/2000
5	1:64	Yes/D5	07/2000
6	1:1024	Yes/D6	07/2000
7	1:256	Yes/D7	07/2000
8	1:64	Yes/D8	08/2000
9-10	1:16	No	-
11	1:64	No	-
12	1:256	No	-
13-25	< 1: 16	No	-

ND: not done; Yes: positive isolation; No: negative isolation.

Table I. – Indirect fluorescent antibody test (IFAT) for toxoplasmosis in dog sera from Minas Gerais, Brazil, and *T. gondii* isolates obtained from muscle samples.

Chicken n°	IFAT titer	Isolation/Isolate name	Month/Year of isolation
1	1:64	Yes/CH1	11/2000
2	1:256	Yes/CH2	11/2000
3	< 1:16	Yes/CH3	12/2000
4	< 1:16	Yes/CH4	04/2003
5	1:1024	Yes/CH5	04/2003
6	1:64	Yes/CH6	04/2003
7	1:16	Yes/CH7	05/2003
8	1:256	Yes/CH8	05/2003
9	1:1024	Yes/CH9	05/2003
10	1:1024	Yes/CH10	05/2003
11	1:1024	Yes/CH11	05/2003
12	1:16	No	-
13	1:64	No	-
14-16	1:256	No	-
17	1:1024	No	-
18-28	< 1:16	No	-

Yes: positive isolation; No: negative isolation.

Table II. – Indirect fluorescent antibody test (IFAT) for toxoplasmosis in free range chickens sera from Minas Gerais, Brazil, and *T. gondii* isolates obtained from muscle samples.

ducible. The isolates D5, D6, CH4, CH5 and CH10 were classified into virulent strains in mice, once all the infected animals died regardless of the tachyzoite concentration. Mice death occurred between six and 23 days after infection. The isolate D8 was classified into non-virulent, as all mice survived 30 days of experiment. All surviving mice showed to have anti-*T. gondii* antibodies (IFAT \geq 1:16). Except for two animals inoculated with a concentration of 10^2 tachyzoites, brain cysts were found in two other animals inoculated with 10^1 tachyzoites and in five other mice inoculated at a concentration of 10^0 tachyzoites. Other isolates (D1, D2, D3, D4, D7, CH1, CH2, CH3, CH6, CH7, CH8, CH9 and CH11) showed to have intermediate virulence traits among the phenotype of virulent strains, such as the strain RH (100 % mortality), and non-virulent strains as ME49 (100 % survival).

Out of the 22 DNA samples directly extracted from chicken hearts, a number of 21 showed amplification products of 241 bp for the region 5' of the *T. gondii* *SAG2* locus and 19 showed an expected amplification product of 221 bp for the region 3' at the same locus. The isolation was positive in eight (38.1 %) of the 21 samples, in which *T. gondii* was identified through nested-PCR. For the three samples with no amplification of the region 3', nested-PCR was repeated at different DNA dilutions, that is, 1:2, 1:10, 1:20 e 1:40, but there was no DNA amplification, precluding RFLP characterization. Of the 21 *T. gondii*-positive samples, 17 (80.9 %) were type I and one (4.8 %) type III. Three samples (14.3 %), with no region 3' amplification, were digested only with the restriction enzyme *Sau3AI*, being characterized as type I or II (Fig. 1). The virulent isolates CH4, CH5 and CH10 were type I. Out of the

Isolates	SAG2	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
D1	I*	0/4**	-	1/4	24.0	5/0	16.0	5/0	17.4
D2	I*	2/1 ^a	16.0	5/0	9.8	5/0	9.6	5/0	7.2
D3	I*	0/5	-	0/5	-	0/5	-	4/1	14.4
D4	I*	4/1	15.5	4/0 ^b	10.5	5/0	8.8	5/0	7.4
D5	I*	5/0	12.6	5/0	10.0	5/0	8.6	5/0	7.0
D6	I*	5/0	10.4	5/0	8.8	5/0	7.0	5/0	6.0
D7	III*	0/0**	-	4/1	28.0	1/4	21.0	4/1	24.75
D8	III*	0/5	-	0/5	-	0/5	-	0/5	-
CH1	III*	0/5	-	1/4	14.0	2/3	15.5	4/1	17.25
CH2	III*	0/5	-	2/3	18.5	3/2	15.7	4/1	15.5
CH3	III*	2/3	13.5	5/0	9.2	5/0	9.0	5/0	7.4
CH4	I	1/0**	18.0	5/0	18.6	5/0	13.2	5/0	9.0
CH5	I	5/0	19.8	5/0	16.2	5/0	12.6	5/0	8.8
CH6	III	0/4**	-	1/3**	17.0	2/3	16.0	2/3	15.5
CH7	I	4/1	24.8	3/2	23.7	4/1	19.0	5/0	17.4
CH8	I	2/2**	13.0	3/1**	13.0	5/0	7.2	5/0	7.0
CH9	I	0/4**	-	3/2	20.0	3/2	21.6	5/0	26.6
CH10	I	5/0	12.2	5/0	10.6	5/0	9.2	5/0	8.6
CH11	I or II	0/5	-	2/3	20.5	5/0	20.6	5/0	14.8

SAG2: analysis on the SAG2 locus of *T. gondii*; *: PCR-RFLP analysis according to Ferreira *et al.* (2006); D/S: dead/surviving mice with positive IFAT; **: some mice were negative by IFAT; d: mean day of death; ^a: three mice inoculated; ^b: four mice inoculated.

Table III. – Virulence comparison for BALB/c mice inoculated by intraperitoneal injection with tachyzoites of different *T. gondii* isolates from the State of Minas Gerais, Brazil.

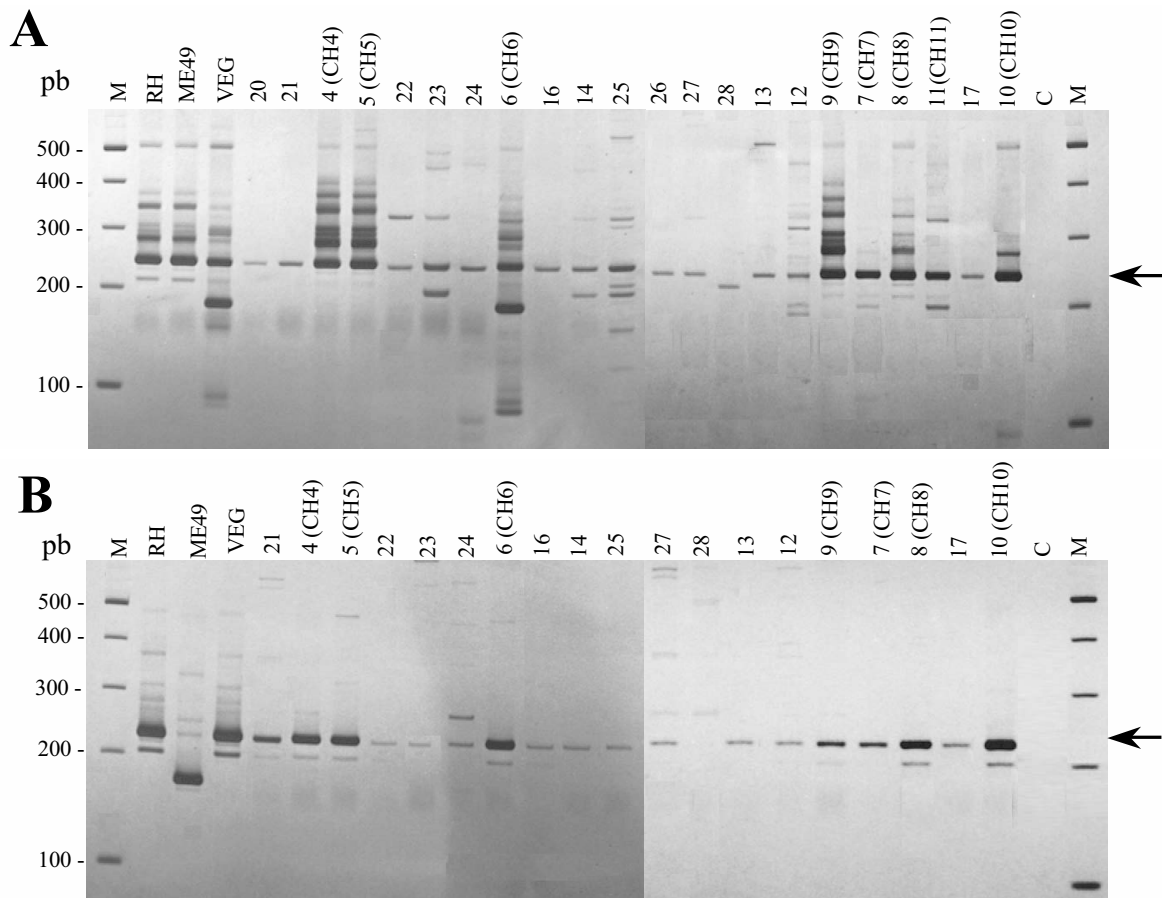


Fig. 1. – RFLP of *T. gondii* SAG2 locus. (A) region 5' with the restriction endonuclease *Sau3AI* (able to distinguish type III from types I and II); and (B) region 3' using the restriction endonuclease *HhaI* (able to distinguish type II from types I and III). Products were resolved in 5 % silver stained polyacrylamide gels for genotyping chicken tissues using the reference strains RH (type D), ME49 (type II) and VEG (type III). M: molecular size marker (Promega 100 pb); C: negative control (no DNA). The arrow indicates restriction polymorphisms.

five isolates of intermediate virulence, one was type III (CH6), three were type I (CH7, CH8 and CH9) and one was type I or II (CH11).

DISCUSSION

T. gondii was not isolated from pig tissues. Serology was carried out using tissue liquids showing to be negative for all animals under study. Such data are not in accordance with the hypothesis that pigs, in Europe and the USA, are the major source of *T. gondii* human infection (Tenter *et al.*, 2000). Recently, investigations have shown that due to the improvements in breeding conditions, including hygiene measures, close confinement and disease prevention, prevalence of *T. gondii* infections in pigs have significantly decreased in the last decade worldwide, including Brazil (Tenter *et al.*, 2000). Those measures may explain the fact that there were no pig isolates in the current work.

T. gondii was isolated from eight out of 25 dogs and 40.9 % showed *T. gondii* antibodies (IFAT \geq 1:16). Mineo *et al.* (2001) reported a prevalence of 36 % of *T. gondii*-seropositive dogs, out of 163 dogs in Uberlândia (MG). In Brazil, dog is not a human food source, but the infection rates may indicate that the parasite and man have been circulating in the same environment (Germano *et al.*, 1985). Moreover, it has been suggested that dogs may act in *T. gondii* mechanical transmission by transporting sporulated oocysts in their fur (Lindsay *et al.*, 1997).

T. gondii was isolated from 11 out of 28 free range chickens and 53.6 % showed *T. gondii* antibodies (IFAT \geq 1:16). Dubey *et al.* (2002), investigating *T. gondii* seroprevalence in free range chickens in São Paulo, Brazil, through Modified Agglutination Test (MAT), reported a rate of 39.02 % and Silva *et al.* (2003), in a similar investigation, reported a *T. gondii* seroprevalence of 64 % for the State of Rio de Janeiro. According to Dubey & Beattie (1988), chickens do not produce *T. gondii* antibodies at levels that can be detected by routine tests, which might explain the fact that, in the present work, *T. gondii* was isolated from two serologically negative chickens. Similarly, Silva *et al.* (2003) obtained *T. gondii* isolates from chicken tissues with negative serology through MAT. High prevalence of *T. gondii* in free range chickens may be explained by their habit to move around freely in direct contact with oocyst-contaminated ground, easily accessed by free-living cats (Literák & Hejlíček, 1993). Our results have shown that, differently from free range chickens, samples from industrialized chicken farms revealed that these animals do not pose a risk to man regarding *T. gondii* infection. Such data are in accordance with those described by Literák & Hejlíček (1993). The

absence of toxoplasmosis infection in animals from industrialized chicken farms is possibly due to the fact that they are close confined under hygiene measures and prevention of diseases, including coccidiosis.

In the current investigation, *T. gondii* isolates were divided into three groups according to the virulence phenotype in BALB/c mice: virulent; non-virulent; and intermediate virulent. The isolates D5, D6, CH4, CH5 and CH10 were classified into virulent, as they led to death of 100 % of mice, regardless of the inoculated concentration, and also produced brain cysts. As a general rule, animals inoculated with such isolates showed a greater survival length as compared to those inoculated with the strain RH. The isolate D8 was classified into non-virulent, similar to the strain ME49, as it caused no death to mice. The great variation observed among the other isolates (D1, D2, D3, D4, D7, CH1, CH2, CH3, CH6, CH7, CH8, CH9 and CH11) has not allowed us to classify them into virulent and non-virulent, being, thus, classified into intermediate virulent. Intermediate virulent strains are believed to be transition strains from the virulent to the non-virulent phenotype (Literák *et al.*, 1998). Guo *et al.* (1997) reported that intermediate virulent strains occasionally occur in nature. However, in the present work, most isolates obtained were classified into intermediate virulent in BALB/c mice.

Out of the 22 DNA samples from chicken heart specimens, a number of 21 specimens had the region 5' of the *SAG2* locus amplified and 19, the region 3'. By genotyping 68 samples using the *SAG2* locus, Fuentes *et al.* (2001) have also remarked difficulties in amplifying regions 3' or 5' for some samples. It is interesting to note that DNA samples extracted from chicken hearts, from which the parasite was isolated, showed to have a greater amount of *T. gondii* DNA, once more intensively stained bands were detected when compared to those samples with no isolates. This little DNA amount has led us to believe that there were few parasites in the tissues; hence, less chance had the mice to be infected by the inoculated material. In accordance with Fuentes *et al.* (2001), nested-PCR using DNA directly extracted from tissue samples has shown to be an accurate and fast method to detect *T. gondii*, which considerably precludes the use of time-consuming techniques, requiring parasite culture. From the results obtained in the present investigation, we may state that the nested-PCR technique is more sensitive to detect the presence of *T. gondii* in tissue samples than the method of inoculation in mice.

Through RFLP of the *T. gondii* *SAG2* locus using DNA directly extracted from chicken heart, 17 samples was identified as type I; one as type III; and three samples as type I or II. Three virulent strains (CH4, CH5 and CH10) in mice were type I and one (CH11) was type I or II. Our results are in agreement with those by

Dubey *et al.* (2002, 2003a), who observed a predominance of *T. gondii* isolates as being type I in chickens obtained in the States of São Paulo and Rio de Janeiro. On the other hand, other reports have revealed that *T. gondii* isolates from chickens obtained in Argentina (Dubey *et al.*, 2003c), Egypt (Dubey *et al.*, 2003d) and the US (Dubey *et al.*, 2003e) were mostly type III, suggesting a different epidemiological prevalence of *T. gondii* genotypes in Brazil, as compared to other countries. Further studies on samples isolated in other Brazilian states are necessary in order to either support or reject such hypothesis.

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