Toxoplasma gondii: evaluation of immunofluorescence assay using heterologous secondary antibody in experimentally infected wild small rodents

Toxoplasma gondii: avaliação da reação de imunofluorescência indireta usando anticorpo secundário heterólogo em roedores silvestres experimentalmente infectados

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Abstract

Toxoplasmosis is a zoonotic infection caused by *Toxoplasma gondii* that affects a wide range of vertebrates. Rodents are intermediate hosts and serve as food for felids, the definitive hosts. However, because of the high variety of species in the order Rodentia, the serological diagnosis is difficult to perform, since the most used techniques require the use of specific antibody conjugated to fluorescein or enzymes. The aim of this study was to evaluate the use of a heterologous secondary antibody conjugate in Immunofluorescence Antibody Test (IFAT) for diagnosis of anti-*T. gondii* antibodies in two species of wild rodents: *Euryoryzomys russatus* (WAGNER, 1848) and *Calomys callosus* (RENGGER, 1830). The specie *Mus musculus* (Linnaeus, 1758) was used as control conjugate. The animals were experimentally infected with five cysts of *T. gondii* (strain ME 49) per animal. The Modified Agglutination Test (MAT), which does not require the use of conjugates, and the presence of *T. gondii* cysts in the rodents were used to confirm the infection. For each animal species, serum samples were collected weekly for five weeks and tested (50 samples per rodent specie, total of 150 samples). None of the samples from *C. callosus* and *E. russatus* were positive in the IFAT when anti-mouse heterologous conjugate was used. Brain cysts of *T. gondii* were microscopically observed in all animals, except in one of the *E. russatus*. Positive results were found in the MAT 14 days after *T. gondii* infection in all three species of rodents and IFAT of the control group (*M. musculus*) was also positive 14 days after infection using anti-mouse (homologous) conjugate. The use of heterologous secondary antibody conjugates should be used with caution and the MAT had a good agreement for serological diagnosis of *T. gondii* in the studied rodent species.

Keywords: Rodents. *Toxoplasma gondii.* Serological diagnosis. IFAT. MAT.

Resumo

A toxoplasmose é uma zoonose causada pelo *Toxoplasma gondii,* que afeta uma grande variedade de vertebrados. Os roedores são hospedeiros intermediários e servem de alimento para felinos, os hospedeiros definitivos. No entanto, por causa da elevada variedade de espécies na ordem Rodentia, o diagnóstico serológico é difícil de ser realizado, uma vez que as técnicas mais empregadas requerem a utilização de um anticorpo específico conjugado com fluoresceína ou enzimas. O objetivo deste estudo foi avaliar o uso de um conjugado secundário heterólogo na Reação de Imunofluorescência Indireta (RIFI) para diagnóstico de anticorpos anti-*T. gondii* em duas espécies de roedores silvestres: *Euryoryzomys russatus* (WAGNER, 1848) e *Calomys callosus* (RENGGER, 1830). A espécie *Mus musculus* (Linnaeus, 1758) foi utilizada como controle do conjugado. As três espécies de roedores foram experimentalmente infectadas com cinco cistos de *T. gondii* (cepas ME 49) por animal. O Teste de Aglutinação Modificado (MAT), que não requer o uso de conjugados, bem como, a presença de cistos de *T. gondii* nos roedores foram usados para confirmar a infecção. Para cada espécie de animal, amostras de soro foram coletadas durante cinco semanas e testadas (50 amostras por espécie de roedor, total de 150 amostras). Nenhuma das amostras de *C. callosus* e *E. russatus* foram positivas na RIFI, quando foi usado o conjugado heterólogo anti-camundongo. Cistos cerebrais de *T. gondii* foram microscopicamente observados em todos os animais, exceto em um dos *E. russatus.* Resultados positivos foram encontrados pelo MAT após 14 dias de inoculação em todas as três espécies estudadas e pela RIFI no grupo controle (*M. musculus*), também no dia 14 após a infecção utilizando conjugado anti-camundongo. O uso de conjugados secundários heterólogos deve ser empregado com cautela e o MAT apresentou uma boa concordância para o diagnóstico sorológico de *T. gondii* nas espécies de roedores estudadas.

Palavras-chave: Roedores. *Toxoplasma gondii.* Diagnósticos sorológicos. RIFI. MAT.

Introduction

Despite being a worldwide distributed disease of great zoonotic potential, the detection of antibodies anti-*Toxoplasma gondii* in wild animals has limitations (FIALHO; ARAUJO, 2003; SOARES et al., 2011). The safer diagnosis for wild and domestic animals is mostly done by isolating the parasite by bioassay in mice (DUBEY; BEATTIE, 1988; DUBEY, 1994). There are some limitations, however, mainly due to the need for fresh tissues from examined animals.

Analysis for the presence of antibodies against this parasite is done through various serological techniques. Among the most used techniques are the Modified Agglutination Test - MAT (DUBEY; DESMONTS, 1987), Enzyme-Linked Immunosorbent Assay - ELISA, and Immunofluorescence Antibody Test - IFAT (CAMARGO, 1973). Most of these tests use antigenic fractions of the whole parasite or soluble antigens (HUGHES; VAN KNAPEN 1982; TOMASI; BARKA; STADTSBAEDER, 1986). However, the use of a gold test to validate results obtained in the serological tests is important and the best test is the isolation of the parasite by mouse bioassay (ROSA et al., 2001).

Among the serological tests, the MAT has many advantages, particularly with respect to the sera of wild animals, with no need for species-specific conjugates and sophisticated devices such as fluorescence microscopy or ELISA reader. However, the technique has some limitations (RUIZ; FRENKEL, 1980; LITERÁK; HEJLÍCEK, 1993; SHAAPAN; FATHIA KHALIL, 2008) and in the case of animals with unknown physiology, false negative results may occur, since this technique is subject to prozone effect (RICHTZENHAIN; SOARES, 2006). The technique also employs toxic reagents like mercaptoethanol, requiring greater care during the procedure (DUBEY; DESMONTS, 1987). Otherwise, the MAT has been described by several authors as the gold standard for detection of anti-*T gondii* antibodies in some species of vertebrates (TÁVORA et al., 2007; VITALIANO et al., 2010). Regarding IFAT, the major problem with this technique is the lack of the fluorescein-conjugated specific antibody commercially available for most wild animals. The use of heterologous conjugates has been tested with satisfactory results for some rodent species (TAFURI et al., 2004; VITALIANO et al., 2005, 2010; SOARES et al., 2011). Assuming that a particular antigen may present different epitopes recognizable to a particular antibody, it is expected that a secondary antibody in the IFAT reaction can recognize regions of the primary antibody common to a group of species phylogenetically related (RICHTZENHAIN; SOARES, 2006).

The goal of this study was to test the feasibility of IFAT for diagnosis of anti-*T. gondii* antibodies in different species of wild rodents by using an anti-mouse commercially available conjugate.

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Material and Methods

Animals - Ten individuals for each of the three rodent species *Euryoryzomys russatus* (Wagner, 1848), *Calomys callosus* (Rengger, 1830) and *Mus musculus* (Linnaeus, 1758), were selected for this study due to the fact that they come from the same superfamily. Founders of these species have been bred in captivity and their offspring used in the experiment. The maintenance of these species in captivity and the use of the rodents have been approved by the Authorization System and Biodiversity Information (SISBio No. 34795-1) and by the Animal Research Ethics Committee from Faculty of Veterinary Medicine and Animal Sciences of the University of São Paulo.

Seven individuals of each specie were infected by intraperitoneal inoculation with five cysts of the ME

49 *T. gondii* strain (DARDÉ; BOUTEILLE; PESTRE-ALEXANDRE, 1992) per animal. Swiss albino mice (*M. musculus*) were used as conjugate positive control. Groups of three animals from each species were not infected and kept as negative controls. The groups were kept individually in polystyrene boxes with autoclaved wood shavings and received commercial food and water *ad libitum*. The boxes were placed on separate stands in ventilated racks. At 7, 14, 21, 28 and 35 days after inoculation (DAI), blood samples were obtained to perform serological tests. Euthanasia of animals was performed with a lethal dose of pentobarbital on the 35th DAI for brain cysts examination.

Modified Agglutination Test - MAT was performed as described by Dubey and Desmonts (1987). *T. gondii* RH strain tachyzoites fixed in formaldehyde solution antigen was used. The sera were tested on 96-well plates with a U-shaped bottom (25 ml of mix antigen plus 25 ml of serum) using a cut off of 1:25 and the positives were tested with a two-fold serial dilution until the final titer. The plates were sealed with plastic film and kept overnight in a chamber at 37ºC. An agglutination reaction (mesh or net) identified the positive sera and a well-defined blue point identified the negative sera. Serum with titer 25 or greater was considered positive (DUBEY, 1997). MAT was kindly supplied by Dr. J.P. Dubey of the United States Department of Agriculture, Beltsville, Maryland, USA.

Immunofluorescence Antibody Test - IFAT was performed as described by Camargo (1973) with some modifications. The anti-mouse IgG conjugate with fluorescein isothiocyanate (FITC-, Sigma-Chemical® Co., St. Louis, MO, USA) was used. Tachyzoites of the RH strain of *T. gondii*, maintained in Swiss albino mouse (*M. musculus*), were used for the antigen production.

The sera obtained from the experimentally infected mouse were diluted in a buffered saline solution (PBS) using an initial dilution of 1:16 (NISHI, 2004). Afterwards, the sera were placed on slides coated with the antigen. *T. gondii* positive and negative mouse sera were added as controls in each slide. The fluorescein-conjugated anti-mouse IgG was diluted in an Evan's blue solution at 10% in a ratio of 1:400.

After drying, the slides were examined in an epifluorescence microscope and those samples that showed tachyzoites completely marked by fluorescence were considered positive and were two-fold serial diluted until the final titer.

Results

None of the serum samples from C. callosus and E. russatus were positive by IFAT (titers ≤ 16) using anti-mouse heterologous conjugate during the experimental period. The same samples were positive from 14th DAI when tested by MAT (titers ≥ 25) (Table 1). The highest titers were found with MAT on 28th DAI in all analyzed species (Table 2).

For *M. musculus,* the IFAT was positive (titers ≥ 16) for four of the seven inoculated mice from 14th DAI and the titers ranged from 16 to 256 (Table 2).

Table 1 - Number of individuals with antibodies anti-*T. gondii* by MAT and IFAT in different species of rodents during the experimental period (35 days) – São Paulo – 2012

Species of Rodents	Days Post – Infection										Presence of Brain
(N =7)	MAT (≥25)					IFAT (≥16)					Cysts N (%)
	7	14	21	28	35	7	14	21	28	35	
E. russatus	0	7	7	7	7	0	0	0	0	0	6 (85.7)
*C. callosus**	0	6	6	6	6	0	0	0	0	0	7 (100.0)
M. musculus	0	6	7	7	7	0	4	3	6	7	7 (100.0)

Note: *One individual died 12 days post-infection. N = Number of animals per specie.

Table 2 - Anti-*T. gondii* antibody titers (number of individuals) detected by the MAT (≥ 25) in *C. callosus, E. russatus* and *M. musculus* and by the IFAT (≥ 16) in *M. musculus* during the experimental period – São Paulo – 2012

	Days Post-Infection				
Rodent species	**7**	**14**	**21**	**28**	**35**
	...	25 (2)	800 (1)	...	...
*C. callosus**#*	< 25 (7)	400 (3)	1600 (1)	25600 (6)	25600 (6)
	...	800 (1)	3200 (1)	...	...
	...	...	6400 (3)	...	...
	...	100 (2)	1600 (1)	...	...
*E. russatus**	< 25 (7)	200 (2)	3200 (2)	6400 (3)	6400 (2)
	...	400 (2)	6400 (3)	25600 (4)	25600 (5)
	...	800 (1)	25600 (1)	...	...
	...	...	50 (1)	...	...
	...	...	100 (1)	3200 (2)	6400 (4)
	...	25 (6)	400 (2)	6400 (2)	25600 (3)
*M. musculus**	< 25 (7)	100 (1)	800 (2)	25600 (3)	
	...	...	1600 (1)	...	...
	...	< 16 (3)	< 16 (4)	< 16 (1)	32 (1)
*M. musculus****	< 16 (7)	16 (4)	16 (2)	64 (1)	64 (1)
	...	...	32 (1)	128 (3)	128 (3)
	...	...	...	...	256 (2)

Note: * Titers of anti-*T. gondii* detected by MAT. ** Titers of anti-*T gondii* detected by IFAT; # One animal died at 12 DAI. Titers of anti-*T. gondii* detected by IFAT for *C. callosus* and *E. russatus* were negative (titers ≤ 16). ... no avaiable.

The controls kept negative by both techniques during the experimental period.

Brain cysts of *T. gondii* were microscopically observed in all animals, except in one of the *E. russatus* (MAT = 25 at 14th DAI and MAT = 800 at 21st DAI). One rodent of the species *C. callosus* died at the 12th DAI with presence of brain cysts and tachyzoites were not observed in peritoneal fluid, lung or liver.

Discussion

There are few data available regarding the use of heterologous or affinity conjugates for detection of anti-*T. gondii* antibodies in sera of wild animals. Vitaliano et al. (2005) found similar results using ELISA with homologous, heterologous (anti-dog conjugate) and affinity conjugates for detection of anti-*T. gondii* IgG in maned wolf – *Chrysocyon brachyurus* (Illiger, 1815). Soares et al. (2011) have also demonstrated that heterologous anti-capybara IgG conjugate in agoutis - *Dasyprocta azarae* (Lichtenstein, 1823) and spotted paca - *Cuniculus paca* (Linnaeus, 1766) can be used for detection of T. gondii antibodies. Tafuri et al. (2004) demonstrated the feasibility of using heterologous anti-rabbit conjugate for detection of antibodies anti-*Leishmania chagasi* by immunohistochemistry in domestic dogs. Mattos et al. (2008), using heterologous anti-dog conjugate, found seropositivity to *Neospora caninum* and *T. gondii* in a significant number (40%) of serum samples of wild canids in captivity. However, none of these authors carried out tests on experimental infected animals to determine the sensitivity of these tests when performed with heterologous conjugates.

In the present study, the use of IFAT using heterologous conjugate was not effective for som e species of rodents and, therefore, the use of heterologous conjugate deserves caution.

In this study, it was used species that are phylogenetically related and belong to the super-family Muroidea.The fact that the two analyzed species were negative for the presence of antibodies against *T. gondii* by the IFAT shows that there is no match between physiologies of these species; what avoids the binding of the conjugate used to IgG IFAT in heterologous species indicates intrinsic differences in their systems. These differences are more common

in neotropical species where there is a high degree of biological diversity. Therefore, it is recommended that the use of serological methods using nonspecific conjugated as secondary antibody should take into consideration the physiological, behavioral, and phylogenetic similarities of the hosts.

For serological tests, the use of heterologous antibodies and antigens always deserves confirmatory tests. Despite MAT demonstrating a high sensitivity and specificity for serological diagnosis of *T. gondii* in wild animals (MINERVINO et al., 2010; MATHEWS et al., 2012), the isolation of the agent by bioassay continues to be the most reliable technique.

The available data about the biology of the relationship between *T. gondii* and wild rodents in Brazil are insufficient. It is estimated that these animals act as intermediate hosts, and possibly participate more significantly in the spread of the disease. Thus, studies on experimental infection of captive individuals are justified by their biological importance. Furthermore, evidence that serological methods can be used accurately is very important to understand the epidemiology of toxoplasmosis in the natural environment.

Calomys callosus has been experimentally studied to determine patterns of *T. gondii* infection (DRESSEN, 1990). Wild rodents are very common in Brazil and these animals are found in all types of forest strata, living in sympatry with synantropic species, such as *M. musculus* and *Rattus rattus* (Linnaeus, 1758) (PAGLIA et al., 2012). In addition, they are highly adapted to captivity. On the contrary, *E. russatus* is rarely used in laboratory studies due to behavioral factors such as aggressiveness, difficulty to adapt to confinement and no social habits that would facilitate its reproduction in captivity (BERGALLO; MAGNUSSON, 2004).

Our results have shown that MAT is the most accurate method for diagnosis of anti-*T. gondii* antibodies in wild rodents and the use of heterologous secondary antibody conjugates in the IFAT should be performed with caution, even among phylogenetically close animals.

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