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ORIGINAL ARTICLE

ISOLATION AND GENOTYPING OF *Toxoplasma gondii* IN SERONEGATIVE URBAN RATS AND PRESENCE OF ANTIBODIES IN COMMUNICATING DOGS IN BRAZIL

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SUMMARY

The role of rodents in the epidemiology of toxoplasmosis was investigated in *Londrina*, *Paraná* State, Brazil. One hundred and eighty-one *Rattus rattus* and one *Mus musculus* were caught in 37 places. Blood and tissues were collected and submitted to the indirect fluorescence antibody test (IFAT) and the bioassay. Serum samples from 61 contacting dogs were also collected. Sixteen rats (8.8%) were positive for *Toxoplasma gondii*, but just two of them were positive by serology and bioassay test. Antibodies were found in nine (4.9%) rats. Tissues of nine rats bioassayed were positive and four isolates were obtained. Restriction fragment length polymorphism (RFLP) analysis was performed using 12 markers (SAG1, SAG2, SAG2-alt, C22-8, C29-2, L358, PK1, BTUB, GRA6, SAG3, Apico, CS3). Genotyping revealed that the four strains isolated from this study have been isolated before in cats and chickens from Brazil. None of the isolates was identified like clonal archetypal T-types I, II, and III. The rats presented lower serologic *Toxoplasma gondii* prevalence (8.8%) compared to contacting dogs (70.5%).

KEYWORDS: Rodents; Toxoplasmosis; Dogs; Genotypic characterization; IFAT.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan that parasites a variety of hosts, from birds to diverse mammals, including humans. The felidae are the final hosts and they are responsible for the shedding of oocysts through feces in the environment. Other animals play a role in the life cycle as intermediate hosts, and in these hosts the protozoa can stay in a latent form represented by tissue cysts[1]. Urban rats are important for the epidemiology of toxoplasmosis acting as a source of infection to domestic cats (definite hosts) and to other carnivores and omnivores such as dogs and pigs[2,3,4].

Sangiorgi[5], in Italy, reported for the first time the presence of *Toxoplasma gondii* in the lungs of an albino laboratory rat. Eyles[6] was the first to look for antibodies against *T. gondii* in wild rats captured in Tennessee, U.S.A. and obtained a seropositivity of 8%. Other researchers have studied the presence of *T. gondii* in rats using different techniques and have found a serological prevalence varying from 0.8% to 59%[6,7,8,9,10,1112,13,14,15]. Studies to isolate the parasite in rodents have found prevalences ranging from 0% to 12.5% in rats captured from different places[16,17,18,19,20].

T. gondii isolated from several animal species, although morphologically undistinguishable, differ with respect to virulence and pathogenicity[21]. Such samples have been characterized based on the virulence established through morbidity and mortality in Swiss albino mice[22]. Nowadays, molecular methods have been adopted to show the existence of genotypes within *T. gondii* species, designated as type I, II, III, who are most prevalent in North America and Europe[23,24,25,26,27]. Recent data suggest a high genetic diversity of *T. gondii* strains in humans and animals from South America[28,29,30,31,32]. A study analyzing 164 isolates from different hosts of South America has grouped the isolates in 42 different genotypes[32]. In Brazil, analysis of isolates from domestic animals has revealed four genotypes which were considered common clonal lineages in the country, called BrI, BrII, BrIII e BrIV[31].

The presence of infection by *T. gondii* in dog populations shows environmental contamination by oocysts eliminated by cats or contamination of food given to these animals. This highlights a possible risk of infection to the human population- as they share the same habitat[33,34,35,36].

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T. gondii genetic variation has high relevance to the understanding of transmission, immunogenicity and pathogenicity, thus the genetic diversity is important to better characterize the molecular epidemiology of toxoplasmosis[37]. Therefore, the aim of this research was to verify the presence of anti-*T. gondii* antibodies in urban sinantropic rodents and communicating dogs in areas of solid residual recycling in *Londrina*, *Paraná* State, and also characterize genotypically the *T. gondii* isolates from rodents.

MATERIAL AND METHODS

Place of study and sampling

Points for collection of animals and samples were areas of solid residual recycling and scrap yards in the five regions in the city of *Londrina, Paraná* State (North, South, East, West and Central). The number of rodents captured was calculated using the EPI6 program[38]. For an infinite population, a prevalence of 50% was estimated, precision of 7.5% and significance level of 5%, resulting in 171 samples. The study was approved by the Ethical Committee in Animal Experimentation of the State University of *Londrina* (n^0. 28/06).

Capture and rodents characterization

Rats were captured between May and December 2006, using cage mouse traps with a trigger activated by bait placed in its interior. Traps were assembled by the end of the afternoon in areas where there were traces of rodents passing by, such as: feces, fat stains, trails, or areas of great offer of food, and checked in the following morning. The species identification was performed based on external morphological characteristics such as the head format and length, tail, ear, posterior foot and body weight; in young animals, we consider that measures could be inferior to that of adults, but the identification was possible due the tail length, posterior foot and head format[39]. The rodents were classified, in the young adult age group, by the presence of well-developed sexual organs[39].

Blood collection from dogs

Blood samples from dogs living in the areas where rodents were captured were collected by puncturing the cephalic or jugular vein using disposable syringes and 25x7 gauge needles. After the coagulum retraction, serum samples were stored in 1.5 mL polypropylene tubes and kept at -20 ºC.

In order to obtain epidemiological data, an epidemiological questionnaire was used, containing data concerning the rodent species, the area in which it was captured, type of trap and bait used, signs of presence of rodents, presence of communicating animals, and the association of these factors was analyzed.

Bioassay

Captured rats were submitted to anesthesia with ethylic ether for the blood collection from the brachial plexus and thereafter the animals were submitted to euthanasia. Serum samples were separated, stored and kept at -20 ºC. In order to conduct the bioassay, fragments from the brain and liver were collected, grounded and homogenized in graal, with the addition of sterile physiological solution containing antibiotic (1,000 UI of penicillin and 100 µg of streptomycin/mL). In order to perform the bioassay, the resulting solution was filtered in sterile gaze and 1 mL was inoculated intraperitoneally for every two Swiss albino female mice with an average weight of 25 g.

Mice were observed daily, and those presenting clinical symptoms were submitted to euthanasia for the collection of peritoneal liquid to check the presence of tachyzoites. In the absence of tachyzoites, the brain, liver and spleen were macerated and inoculated in two other mice. Six weeks after inoculation, asymptomatic animals were euthanatized, their blood was collected for serology and brain fragments were collected for the observation of tissue cysts. Mice were considered negative to *T. gondii* in the absence of parasites (tachyzoites or tissue cysts) and of specific serum antibodies[17,40].

Serology

Serum samples from dogs, captured rats and bioassayed mice were submitted to the indirect fluorescence IgG anti-*T. gondii* antibodies test (IFAT), using tachyzoites of the RH strain as antigen[41]. Species-specific fluorescein isotiocianate-labeled conjugates were used (Sigma Chemical Co. and Zimed) as well as positive and negative sera controls. Positive dilutions were ≥ 1:16 for both species[2,40].

In order to obtain positive controls for the genus *Rattus,* three bi-monthly inoculations with live tachyzoites from the RH strain were performed intraperitoneally, in three albino *R. norvegicus*. The first inoculum contained 2 x 10^3 tachyzoites/ mL, the second and third, 1 x 10^6 tachyzoites/ mL. The rats did not present any clinical signs and were euthanatized 15 days after the third inoculation for the collection of blood. Serum samples used as negative controls were from albino *R. norvegicus*, prepared at the Central Vivarium of the State University of *Londrina*. After standardization, the titer of positive serum samples controls and conjugate were 1,024 and 300, respectively.

DNA extraction

DNA extraction was performed using tachyzoites obtained from peritoneal washes of mice according to Garcia *et al*.[42]. Briefly, the sample was homogenized, 300 µL were transferred to a microtube with the same volume of the extraction buffer (200 mM of NaCl, 20 mM of Tris, 50mM of EDTA, proteinase K 1mg/ mL and 2% SDS) and incubated at 56 ºC for 1h. After this, 300 µL of tamponed phenol were added and centrifuged at 13,000 *x g* during 5 minutes. The aqueous phase was transferred to another tube containing phenol: chloroform: isoamilic alcohol and centrifuged at 13,000 *x g* during 5 minutes. The DNA precipitation was performed using sodium acetate and ethanol[43].

Polimerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP)

In order to determine the *T. gondii* genotypes, 12 markers SAG2[44], GRA6[45], CS3[31], SAG3[46], SAG1, SAG2-alt, C22-8, C29-2, L358, PK1, BTUB, Apico[47] were used. The first amplification followed a previously described protocol and then a nested PCR was performed[29]. Eight *T. gondii* reference strains (GT1, PTG, CTG, TgCgCa1, MAS, TgCatBr5, TgCatBr64 and TgRsCr1) were used as controls. In case one or more markers were not amplified, isolates would be subjected to a 18S rDNA amplification, to exclude the possibility of other apicomplexan parasites closely related to *T. gondii* (*Hammondia hammondi, Neospora caninum* and *Sarcocystis neurona*)[48].

Statistical analyses

For the tabulation of data obtained from the epidemiological questionnaires and from the bioassay and serology, the EPI6 software was used and the Chi-square test, the Fisher Exact test and the *Odds*

Ratio (OR) with a confidence interval of 95% (IC 95%) were used. The significance level was established at 5%.

RESULTS

A total of 182 rodents were captured from 37 areas of storage and recycling of solid residuals. In 27 of these areas, there were communicating dogs, whose blood samples were also collected. There were also residences in 15 of these 37 places.

A total of 181 (99.4%) rodents captured belonged to the species *Rattus rattus* and one (0.6%) to the species *Mus musculus*, being 77 (42.3%) male and 105 (57.7%) female animals. One hundred and fifty rodents (82.42%) were classified as adults and 32 (17.58%) as young. Sixteen (8.8%) were positive for *T. gondii*, all *R. rattus.* In the bioassay, four strains were isolated, two from rats that yielded positive IFAT and two from IFAT-negative rats. Serological (IFAT) and bioassay results are presented in Table 1. The presence of anti-*T. gondii* antibodies was more frequent in male rats ($p = 0.048$; OR = 3.33; CI 95%;1.00 – 11.73). There was no significant difference regarding the region where the rats were captured within the city of *Londrina* ($p = 0.106$), nor with respect to the age group ($p = 0.670$). Among seven serological negative rats which were positive by bioassay, six were adults weighting between 150 and 250 g and one was young, weighting approximately 50 g.

Three of four samples from which the parasites were isolated (bioassay), mice presented clinical signs of toxoplasmosis approximately ten days post inoculation (d.p.i), followed by death. Few tachyzoites were recovered from the peritoneal cavity. Regarding the fourth sample, mice presented clinical signs of toxoplasmosis at 24 d.p.i. They were then euthanized and tachyzoites were recovered from the peritoneum.

Concerning the 61 partly-domiciled dogs examined, 50 (82.0%) did not have any defined breed and 27 (44.3%) were male. From the 43 dogs that had their ages informed, 14 (32.6%) were younger than one year old. The prevalence of positive IFAT in dogs was 70.5% (43/61) with titers of 1/16 (27.9%), 1/64 (34.9%), 1/256 (25.6%) and 1/1024 (11.6%). There was a significant difference regarding the age ($p = 0.035$), and the animals ≤ 1 year old presented OR = 0.21 (CI 95%; 0.04 – 1.05). There was no difference with respect to the gender ($p = 0.406$) and the breed ($p = 1.00$).

Table 1

Results of anti-*Toxoplasma gondii* antibody titers obtained by IFAT in rodents captured in solid residual recycling areas, *Londrina – Paraná* State, (2006). IFAT and bioassay results in mice used in the bioassays

Rats	IFAT	Bioassay (Swiss albino mice)	
Sample	Titers	Titers (IFAT)	Isolate (tachyzoites)
25	-	16	-
28	16	-	-
48	32.000	-	-
60	-	-	+
61	-	-	+
134	4.096	-	-
143	256	-	+
146	4.096	-	+
155	1.024	-	-
164	16	-	-
168	16	-	-
172	16	-	-
108	-	16	-
185	-	64	-
187	-	16	-
189	-	16	-

The analysis of the four *T. gondii* isolates with twelve genetic markers demonstrated a genetic diversity, classifying these isolates as atypical genotypes. The four isolates are genotypically identical to two strains (Table 1) originally isolated from cats in *Paraná*, Brazil[49]. In all of the isolates, the amplification of the twelve markers was successful, so that the 18S rRNA amplification was not used for differentiation. The four rats in which *T. gondii* was isolated had weights between 150 and 200 g and had reproductive organs well defined, being classified as adults.

Table 2

Multilocus genotyping of *Toxoplasma gondii* strains isolated from urban rats, *Londrina*, PR, 2006

Isolate	Markers												# Toxo DB	PCR-RFLP Genotype	Genotypes References
	SAG1	5’-3’-SAG2	alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	CS3			
60	I	III	III	III	III	III	I	I	I	III	III	II	# 21	TgCatBr10, 22, 23, 28, 31, 32, 37, TgCkBr95, TgCpBr29, TgRrBr09	55, 49, 29, 47, 64
61	I	III	III	III	III	III	I	I	I	III	III	II			
143	I	III	III	III	III	III	III	I	III	III	III	II	# 14	TgCatBr15, TgCkBr82, 90, 153, TgCkCh1, TgCkCo2,TgCkVe3, TgCtCo14, TgCyW6, TgDgBr19, TgDgCo9, 12, 15, 18,	65, 66, 49, 67, 68 29, 69, 29, 32, 47
146	I	III	III	III	III	III	III	I	III	III	III	II			

DISCUSSION

There was a predominance of the *R. rattus* species in the city of *Londrina*, which was already expected, since this species has shown a huge dispersion in the urban environment, due to the increase of high buildings and with false ceilings, technical galleries for passage of wires and cables allowing the existence of shelters. Despite the lack of statistical significance, male rats were more prevalent probably due to more exploratory habits related to the male gender[39]. In natural areas, rodents are infected by *T. gondii* mainly by the ingestion of sporulated oocysts present in the environment or transplacentary (congenital transmission)[50]. Rats are one of the most resistant animals to *T. gondii*, clinical manifestation is associated to immunosuppressant factors, the type of strain, the amount of inoculum, the age of rats and the infecting form of *T. gondii*[51].

Ito *et al.*[52] observed that rats fed with *T. gondii* oocysts from different isolates have seroconverted without presenting clinical signs, but later the presence of tissue cysts was demonstrated in several organs, mainly in the brain. Dubey[53] showed that adult rats inoculated with a few oocysts presented subclinical infection, but developed anti-*T. gondii* antibodies 29 d.p.i., with an increase in serological titers up to 75 d.p.i.

The prevalence of toxoplasmosis (8.8%) found in rodents in the city of *Londrina* is compatible with some studies conducted worldwide. Franti *et al.*[12] in the State of California, USA, observed a seroprevalence of 4% in 160 urban rats, and of 38% in 47 cats. Mercier *et al.*[54] described a prevalence of 1.96% in 766 domestics and peridomestic rodents in Niamey, Niger. In Brazil, some studies reported lower prevalences in urban sinantropics rodents; in *Umuarama*, *Paraná* State, none of 24 *R. rattus* and 19 *Mus musculus* showed positive results in the sorological tests[55], and in *São Paulo,* Muradian *et al.*[56] established the prevalence of 0.46% in 217 captured rodents by means of bioassays. Higher prevalences were found in Costa Rica (30.4% *Rattus*), in Panama (23.3% *R. norvegicus*), England (59% *M. domesticus*) and Pakistan (58.57% *R. rattus*; 36.66% *M. musculus*)[9,13,18,57]. In these studies, it is clear that both, theserological prevalence and the isolation of *T. gondii* in rats are lower than in other domestic species, and these results are compatible with those we found, as the prevalence in rats was 8.8% and in communicating dogs was 70.5%.

In this study, four rodents had *T. gondii* isolated, fact that evinced the importance of the bioassay to characterize the animals as a source of infection for predators such as dogs and cats. Dubey *et al.*[16] studied the prevalence and genotyping of *T. gondii* in rats captured in Grenada, West Indies (Caribbean), from a total of 238 *R. norvegicus,* two (0.8%) were positive by the modified agglutination test (MAT $\geq$ 1:40) and there was only one isolation of *T. gondii* genotype III. Researchers have concluded that rats were not important to the epidemiology of toxoplasmosis in that island. Other studies conducted in Brazil also revealed low frequencies of *T. gondii* isolation in rats. Muradian *et al.*[56] obtained one isolate from *R. norvegicus* captured in *São Paulo*. Araújo *et al.*[55] obtained two isolates from *R. rattus* captured in *Umuarama, Paraná*, and afterwards they also obtained isolates from serum-negative animals as we did in our study. This could happen when the animal is infected early in the intrauterine life, through a vertical transmission. In this case the animals show non measurable antibody levels[58]. Dubey[53] showed that congenitally infected rats presented brain cysts after two months of age. During this time, some of the animals were serologically negative. Such a result has led to the conclusion that isolation of *T. gondii* is possible in rats with negative serology and that the proportion of infected rats with no detectable antibodies is unknown in the environment. This explains the fact that, in the present study, there were seven negative rats by serology that were positive in the bioassay. Of these, six rats were characterized as young adults, therefore the absence of antibodies could be attributed to a recent infection.

The prevalence of *T. gondii* in dogs that lived in the areas where the rats were captured is compatible with the one found in other studies conducted in the *Paraná* State ranging from 19.7% to 75.98% in dogs from urban areas and from 20.8% to 84.1% in dogs from rural areas[33,34,35,36]. In this study we should also associate the difference in the prevalence of toxoplasmosis observed among rats and dogs, with the fact that the predominant rodent population in the city was of *R. rattus*; a species that presents arboreal habits and is only found in the ground when searching for food. The exposure to environments contaminated by oocysts is greater for dogs, that are frequently semi-domiciled and have a major dispersion area than rats; in addition they have a longer life span when compared to rodents. This exploratory habit of dogs also clarify the low prevalence in less than one year-old dogs comparing with older dogs, considering that older dogs have already had more chances of contact with *T. gondii* infectant forms in the environment.

Domestic cats are the main responsible for the environmental contamination and probably for the prevalence of toxoplasmosis in dogs in urban areas. The hunting of rats and birds is the main form of infection in cats, and the vital cycle induced after the ingestion of tissue cysts is the most efficient, since 97% of cats eliminate oocysts in a pre-patent period of three to ten days[2,11]. In Brazil, the prevalence of infection by *T. gondii* in cats is high, varying from 18% to 84.4%[33,49,59,60,61,62,63].

Genotyping isolates of *T. gondii* by PCR-RFLP with multiple markers has a resolution similar to DNA sequencing, being simpler and presenting a lower cost[47]. In this study, all markers were amplified in the four isolates, but they were not characterized as belonging to the three clonal lineages (I, II, III), usually found in North America and Europe. Nevertheless, the four genotypes are similar to those found in other studies carried in Brazil, with cats, chickens, goats, capybara, rats and dogs (Table 2). Among the four isolated strains, three showed virulence in mice, as they showed clinical signs of *T. gondii* infection ten d.p.i followed by death. These results are in accordance with the study carried by Pena *et al.*[31] who showed that the genotype II in the CS3 marker is related to a higher virulence in mice. One of the isolates showed a low virulence, the mice presented signals of infection 24 d.p.i, despite the same genotype. Nevertheless, is well known that *T. gondii* virulence in mice does not depend only on the parasite lineage, but also to the infectious inoculum, the route of infection, and the parasite form[31].

The dominant rodent species in the solid residual storage and recycling area in the city of *Londrina* was *R. rattus*, which presents mainly arboreal habits. Prevalence of toxoplasmosis in captured rodents was lower than in dogs, however the prevalence found in dogs inhabiting the same area indicates the environmental contamination by oocysts, or the contamination of food given to these animals. In our study, the use of genotyping tools for the identification of isolates allowed the characterization of strains, that had been previously found in different

Brazilian animals. The parasite isolation from serological negative animals by the IFAT has demonstrated that the identification of antibodies in those animals is not sufficient to characterize these animals as sources of infection for cats.

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