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TOXOPLASMA GONDII INFECTIONS IN CATS FROM PARANÁ, BRAZIL: SEROPREVALENCE, TISSUE DISTRIBUTION, AND BIOLOGIC AND GENETIC CHARACTERIZATION OF ISOLATES

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ABSTRACT: Cats are important in the epidemiology of *Toxoplasma gondii* because they are the only hosts that can excrete environmentally resistant oocysts. The prevalence of *T. gondii* was determined in 58 domestic cats from 51 homes from Santa Isabel do Ivai, Paraná State, Brazil where a water-associated outbreak of acute toxoplasmosis had occurred in humans. Antibodies to *T. gondii* were found with the modified agglutination test in 49 of 58 (84.4%) cats at a serum dilution of 1:20. Tissues (brain, heart, and skeletal muscle) of 54 of these cats were bioassayed in *T. gondii*–free, laboratory-reared cats; *T. gondii* oocysts were excreted by 33 cats that were fed feline tissues. Brains from these 54 cats were bioassayed in mice; *T. gondii* was isolated from 7. Skeletal muscles and hearts of 15 cats were also bioassayed in mice; *T. gondii* was isolated from skeletal muscles of 9 and hearts of 13. The results indicate that *T. gondii* localizes in muscle tissue more than the brains of cats. In total there were 37 *T. gondii* isolates from 54 cats. Most isolates of *T. gondii* were virulent for mice. Genotyping of the 37 isolates of *T. gondii*, using the SAG2 locus, revealed that 15 isolates were type I and 22 were type III. The absence of type II genotype in cats in this study is consistent with the previous studies on *T. gondii* isolates from Brazil and is noteworthy because most *T. gondii* isolates from the United States are type II. These findings support the view that Brazilian and North American *T. gondii* isolates are genetically distinct. This is the first report of genotyping of *T. gondii* isolates from the domestic cat.

Toxoplasma gondii infections are widely prevalent in human beings and animals worldwide (Dubey and Beattie, 1988). Humans become infected postnatally mainly by ingesting tissue cysts from undercooked meat or from the food or drink contaminated with oocysts shed in cat feces. Cats are important in the natural life cycle of *T. gondii* because they are the only hosts that can directly spread *T. gondii* in the environment. Cats can ''recycle'' and amplify the infection by releasing millions of infective units into the environment. Recently, an outbreak of clinical toxoplasmosis in humans was epidemiologically linked to drinking water from a municipal water reservoir in Santa Isabel do Ivai, Paraná State, Brazil (J. P. Dubey, unpubl. data). The objectives of this study were to determine prevalence of *T. gondii* in cats from this town and characterize isolates of *T. gondii* from these cats.

MATERIALS AND METHODS

Naturally infected cats

In total, 58 adult (22 males and 36 females) cats were obtained from 51 homes within a 3-km area around Santa Isabel do Ivai (latitude 23°00′00″, longitude 53°11′50″), Paraná State, Brazil. The cats were purchased, and transported to the Veterinary Hospital, Londrin State University, where they were humanely killed. The cats were obtained in 3 batches of 20, 23, and 15 in October 2003, December 2003, and January 2004, respectively (Tables I–III). From each cat, serum, brain, and whole heart were obtained for *T. gondii* examination. In addition, muscles (15–25 g) from limbs were obtained from cats in batch nos. 2 and 3. The tissues were transported cold by air from Brazil to the U.S. Department of Agriculture's, Beltsville, Maryland, laboratory, where all *T. gondii* evaluations were performed. Four to 5 days elapsed between killing and examination for *T. gondii*, and during this time samples were kept cold.

Serologic examination

Sera from cats were diluted 2-fold starting at 1:10 dilution and assayed for *T. gondii* antibodies with the modified agglutination test (MAT) as described (Dubey and Desmonts, 1987).

Bioassay of cat tissues for *Toxoplasma gondii*

Bioassay in cats: Tissues of 54 of 58 cats (Tables I–III) were bioassayed in *T. gondii*–free cats as described (Dubey, 1995) Cats were selected for this purpose because they are the most sensitive hosts for the detection of *T. gondii* in tissues (Dubey, 2001). For the cat bioassay, tissues from each of 49 seropositive cats from Paraná were pooled and fed to 49 cats. Tissues from 5 seronegative cats were fed to 3 cats (Tables II, III); tissues from 4 seronegative cats from batch no. 1 were not bioassayed. Feces of cats were examined for shedding of *T. gondii* oocysts 3–14 days after feeding feline tissues as previously described (Dubey, 1995). Oocysts obtained by cat bioassay were allowed to sporulate for 1 wk at room temperature, and sporulated oocysts were fed to 2 mice each. In addition, fecal samples without microscopically visible oocysts from cats fed tissues from batch nos. 2 and 3 were bioassayed in mice. Tissues from the mice fed oocysts were inoculated subcutaneously (SC) in to another 2 mice to confirm their identity as *T. gondii* (Dubey and Beattie, 1988).

Bioassay in mice: In addition to the cat bioassay, feline tissues were bioassayed in mice (Dubey and Beattie, 1988). From each cat in batch nos. 1 and 2, 5 g of brain was homogenized in 50 ml of saline (0.85% NaCl), centrifuged at 1,200 *g*, and the sediment suspended in 5–10 ml of saline containing antibiotics and inoculated SC into 5 mice. Tissues from cats in batch no. 3 were bioassayed individually in mice after digestion in pepsin (Dubey, 1998). For this, each tissue was homogenized in 5 volumes (w/v) of saline, mixed with 5 volumes of acidic pepsin, and the mixture was incubated in a shaker water bath for 1 hr at 37 C. The digest was centrifuged, neutralized, mixed with antibiotics, and the homogenate was inoculated SC in to 5 mice. The mice used were Swiss Webster albino females obtained from Taconic Farms, Germantown, New York.

Tissue impression smears of mice that died were examined for *T. gondii* tachyzoites or tissue cysts (Dubey and Beattie, 1988). Survivors were bled on day 45 postinoculation (PI), and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT.

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TABLE I. Isolation of *Toxoplasma gondii* from naturally infected cats from Paraná, Brazil (batch no. 1).

Cat no., sex	MAT titer	Isolation by		Virulence to mice‡	Genotype	Isolate designation
		Cat bioassay*	Mouse bioassay†			
1, M	200	No	0/5			
2, F	200	Yes	0/5	Virulent	I	TgCat Br1
3, F	400	Yes	0/5	Virulent	I	TgCat Br2
4, F	200	No	0/5			
5, M	800	Yes	0/5	Nonvirulent	III	TgCat Br3
6, M	25	Yes	0/5	Nonvirulent	III	TgCat Br4
7, M	800	No	0/5			
8, F	1,600	Yes	0/5	Virulent	III	TgCat Br5
9, F	200	Yes	0/5	Virulent	III	TgCat Br6
11, F	200	Yes	0/5	Virulent	I	TgCat Br7
13, F	50	No	0/5			
15, F	400	No	0/5			
17, F	200	No	0/5			
19, M	800	No	0/5			
20, M	1,600	No	1/5§	Virulent	I	TgCat Br8
21, F	400	Yes	0/5	Virulent	I	TgCat Br9
12, M; 14, M; 16, F; 18, F	<10	Not done				

* Shedding of oocysts by recipient cats fed feline brain and heart.
† No. of *T. gondii* positive/no. of mice inoculated with 5 g of cat brain.
‡ Based on infectivity of tachyzoites obtained from oocyst infection.
§ Mouse died of acute toxoplasmosis 11 days PI and the isolate was virulent to the subpassaged mice.

TABLE II. Isolation of *Toxoplasma gondii* from naturally infected cats from Paraná, Brazil (batch no. 2).

Cat no., sex	MAT titer	Isolation by		Virulence‡	Genotype	Isolate designation
		Cat bioassay*	Mouse bioassay†			
1, F	20	Yes	0/5	Virulent	III	TgCat Br10
5, F	100	No	0/5			
7, M	400	Yes	0/5	Virulent	III	TgCat Br11
9, F	20	Yes	0/5	Virulent	I	TgCat Br12
11, F	400	Yes§	0/5	Virulent	III	TgCat Br13
12, F	20	No	0/5			
13, M	80	Yes§	0/5	Virulent	III	TgCat Br14
14, F	100	Yes	0/5	Virulent	III	TgCat Br15
15, F	800	No	0/5			
16, M	400	No	0/5			
17, M	100	No	0/5			
18, F	1,600	Yes	0/5	Virulent	III	TgCat Br16
20, F	800	Yes	5/5¶	Virulent	I	TgCat Br17
21, M	400	No	0/5			
22, F	800	Yes	0/5	Virulent	III	TgCat Br18
23, F	100	Yes	0/5	Virulent	I	TgCat Br19
24, F	>6,400	Yes	0/5	Virulent	III	TgCat Br20
25, F	3,200	Yes	5/5¶	Virulent	I	TgCat Br21
26, M	800	Yes	0/5	Virulent	III	TgCat Br22
2, F; 4, M; 6, M	<10	No	0/5, 0/5, 0/5			
10, F	<10	No	0/5			

* Shedding of oocysts by recipient cats fed feline brain, heart, and muscle.
† No. of mice positive for *T. gondii*/no. of mice inoculated with 5 g of undigested cat brain.
‡ Based on infection with tachyzoites obtained from oocyst infection.
§ Oocysts were not detected by microscopic examination but were revealed by mouse bioassay.
¶ Mice died 10–13 days PI of acute toxoplasmosis.

TABLE III. Isolation of *Toxoplasma gondii* from naturally infected cats from Paraná, Brazil (batch no. 3).

			Mouse bioassay†						
			Brain		Heart		Skeletal muscle		
Cat no., sex	MAT titer	Cat bioassay*	No. of mice dead (day of death)	No. infected	No. of mice dead (day of death)	No. infected	No. of mice dead (day of death)	No. infected	Genotype§ (isolate designation)
1, F	80	Yes	0	0	3 (14–21), ‡ S	5	5 (12–21)	5	I (TgCat Br23)
2, F	80	No	0	0	5 (13–14)	5	5 (10)	5	I (TgCat Br24)
3, F	80	Yes	0	0	5 (19–24)	5	1 (19)	1	III (TgCat Br25)
4, M	80	Yes	4 (17–27), S	5	0	0	0	0	I (TgCat Br26)
5, F	640	Yes	5 (14)	5	5 (14–21)	5	5 (12–14)	5	III (TgCat Br27)
6, F	320	Yes	0	0	0	1	0	0	III (TgCat Br28)
7, F	<10	No	0	0	1 (39)	1	0	0	III (TgCat Br29)
8, M	160	Yes	5 (12–13)	5	5 (12)	5	5 (10–12)	5	I (TgCat Br30)
9, M	80	Yes	3 (18–21)	3	4 (16–28)	4	5 (13)	5	III (TgCat Br31)
10, F	80	Yes	0	0	1 (19), S, S, S	4	5 (13)	5	III (TgCat Br32)
11, F	80	No	0	0	2 (19, 24)	2	0	0	III (TgCat Br33)
12, F	160	Yes	0	0	5 (14)	5	2 (21–28)	2	I (TgCat Br34)
13, F	80	Yes	0	0	5 (13–14)	5	5 (10–19)	5	I (TgCat Br35)
14, M	80	Yes	0	0	0	0	0	0	III (TgCat Br36)
15, F	160	Yes	0	0	3 (18–24)	3	0	0	III (TgCat Br37)

* Shedding of oocysts by recipient cats fed feline tissues.
† Five mice per cat tissue.
‡ S=Mice survived for 50 days PI and tissue cysts were found in the brain.
§ Genotypes were determined from isolates obtained from heart, except the isolates from cat no. 4, which was from the brain and from cat no. 14, which was from the oocysts.

Mice were killed 50 days PI, and their brains were examined microscopically for tissue cysts, and a portion of the brain was frozen for DNA extraction. Mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were demonstrable in their tissues.

Genetic characterization

Toxoplasma gondii DNA was extracted from infected mouse tissues as described by Lehmann et al. (2000). Polymerase chain reaction–restriction fragment length polymorphism (RFLP) genotypes of SAG2 locus were used to determine the genetic type (Howe et al., 1997).

Examination of cats associated with water reservoir

The outbreak of toxoplasmosis in humans occurred from November 2001 to January 2002 (J. P. Dubey, unpubl. data). The city was served by 2 underground water cisterns (A and B). Cistern A was epidemiologically associated with the outbreak, and *T. gondii* was isolated from water samples served by cistern A. The cisterns were not completely sealed, and the water was not filtered but chlorinated before human consumption. A cat living near the cistern-A area delivered 3 kittens in October 2001. The kittens were living on the top of the water cistern and were probably weaned in early November 2001. One of these kittens (cat X) was trapped and blood obtained for examination for antibodies to *T. gondii* by the indirect fluorescent antibody test; titers were 1:64 on 25 January and 1:1,024 on 30 January 2002, indicating a recently acquired *T. gondii* infection. This cat was killed on 24 January 2003, and samples of liver, lung, spleen, and muscles were fed to a laboratory-raised cat (cat Y). Cat Y shed oocysts 5 days later; these oocysts were sent from Brazil to Beltsville in March 2003 for further characterization.

RESULTS

Antibodies to *T. gondii* were found in 49 of 58 (84.4%) cats, in titers of 1:20 or 25 in 4, 1:40 or 1:50 in 1, 1:80 or 1:100 in 14, 1:160 or 1:200 in 9, and 1:320, or more in 21 cats (Tables I–III).

Toxoplasma gondii was isolated from 9 of 20 cats belonging to batch no. 1, of which 8 were by bioassay in cats and 1 by bioassay in mice (Table I). Seven of the 9 isolates were virulent for mice. Genetically, 4 of the *T. gondii* isolates were type III and 5 were type I (Table I).

Toxoplasma gondii was recovered from 13 of 23 cats from batch no. 2. Oocysts were seen microscopically in the feces of 11 cats and were detected in feces of 2 additional cats by mouse bioassay (Table II). The parasite was also isolated by bioassay of brain tissue of 2 cats. All 13 isolates were virulent for mice. Genetically, 9 isolates were type III and 4 were type I.

From cats in batch no. 3, *T. gondii* oocysts were detected microscopically in the feces of 12 cats (Table III). One of the 3 cats whose tissues were not infective to cats had no antibodies to *T. gondii* (Table III). Viable *T. gondii* was isolated by mouse bioassay from tissues of 14 of 15 cats; of these, 3 isolates were from all organs, 7 were from heart and muscle, 3 were from heart alone, and 1 was from brain alone (Table III). Most (95%, 99 of 105) mice that became infected with *T. gondii* after inoculation with feline tissues died of toxoplasmosis between 11 and 29 days PI, irrespective of the genotype (Table III). In total, *T. gondii* was isolated from all 15 cats in batch no. 3. The genotype of the 15 isolates of *T. gondii* was type I in 6 and type III in 9 (Table III).

All 3 mice fed oocysts transported from the cistern-A region in Brazil became ill and 1 was killed on day 4 PI and 2 died on day 8 PI; tachyzoites were found in their mesenteric lymph nodes. Mice inoculated SC with tachyzoites of this isolate (designated TgCat Br38) died 6–8 days PI; tissues from these mice were fed to a laboratory-raised cat at Beltsville to determine if cats fed acutely infected mice will shed oocysts. This cat shed oocysts 4 days PI; the mice fed sporulated oocysts from this

cat died 4 days later. The DNA obtained from the mesenteric lymph node of the mouse that died 4 days PI was type I.

DISCUSSION

Determination of antibodies and isolation of the parasite are better measures of the prevalence of *T. gondii* in cats than fecal examination for oocysts as only few (1%) cats shed oocysts at any given time (Dubey and Beattie, 1988). However, there are no data on validation of serological tests for the detection of *T. gondii* in naturally infected cats. In experimentally infected cats, MAT was the most sensitive assay for the detection of *T. gondii* antibodies; antibodies were detected within 3 wk of feeding tissue cysts and persisted in high titers for 5 yr even in the absence of reinfection (Dubey and Thulliez, 1989; Dubey, 1995; Dubey et al., 1995). In addition, antibodies to *T. gondii* were not detected in 1:25 dilution in more than 1,000 laboratory-raised cats that are commonly infected with a related coccidium, *Isospora felis* (J. P. Dubey, unpubl. data). Antibodies to *T. gondii* were also absent in cats that are infected with *Toxocara canis*, *Ancylostoma caninum*, *Isospora rivolta*, *Mycobacterium tuberculosis* and various viruses (Dubey, 1978; J. P. Dubey, unpubl. data). Therefore, the MAT appears to be specific for *T. gondii*.

Ruiz and Frenkel (1980) made an extensive study of *T. gondii* infection in 237 cats from 7 localities in Costa Rica. Sabin–Feldman dye test antibodies were found in 109 (46%) and *T. gondii* oocysts were detected in feces of 55 (23%) cats; in 8.4% with antibodies and in 14.8% without antibodies. It is of interest that the number of oocysts was so low that they were detected microscopically in only 12.7% of the infected cats and in the remaining cats (87.3%), only by bioassay in mice. Most of the cats that were shedding oocysts were less than 3 mo old, and 48% of cats had become infected by 2 mo of age (Ruiz and Frenkel, 1980). Unlike the study in Costa Rica, all cats in this study were adults (approximately 2 yr old) and 84% were seropositive. In experimental infections, cats stop shedding *T. gondii* oocysts by the time they seroconvert (Dubey and Frenkel, 1972). Because most cats in this study were seropositive, they had probably ceased shedding oocysts. Therefore, no attempt was made to bioassay feces from these cats.

In this study, *T. gondii* was isolated from 36 of 49 seropositive and from 1 of 4 seronegative cats. The seronegative cat found to be infected with *T. gondii* might have been recently infected. These data add to the validity of MAT in cats. The success of isolation of *T. gondii* in the 3 batches of cats varied, probably related to the methods of bioassay. In batch no. 1, brains and hearts were fed to cats, and feces of only those cats with microscopically confirmed oocysts were bioassayed in mice. Thus, it is possible that some infected cats in batch no. 1 might have been missed. Whereas, cats from batch no. 2 were also fed skeletal muscles, and feces of all cats were bioassayed in mice and 2 additional infected cats could be detected.

Little is known about the tissue distribution of *T. gondii* in cats. *Toxoplasma gondii* is considered to have an affinity for encystment in neural tissue, but this assumption is based on infections in mice. Seventeen tissues from each of 7 cats fed *T. gondii* tissue cysts, 143–473 days earlier, were bioassayed in mice (Dubey, 1977); *T. gondii* was isolated from the hearts of 5, skeletal muscle and spinal cords of 4, and brains of only 3 cats. In another study, distribution of *T. gondii* was studied in 9 cats fed 100–100,000 oocysts and killed 37 or 51 day PI; *T. gondii* was isolated from the tongues of all 9, hearts of 5, brains of 4, and eyes of 1, irrespective of the dose of oocysts administered (Dubey, 1997). In the present study, *T. gondii* was isolated from the brains of only 3 of 21 cats in batch nos. 2 and 3 that were proved to be infected with *T. gondii*. These results indicate that the density of *T. gondii* in brains of cats is low because a homogenate of 5 g of brains was bioassayed in mice. From cats in batch nos. 1 and 2, the brains were not digested in pepsin because we wanted to estimate the density of *T. gondii* in cat brain to attempt microisolation of tissue cysts for future genetic studies. Because of the low density of *T. gondii* in cat brain in batch nos. 1 and 2, tissues from cats in batch no. 3 were digested in pepsin to concentrate *T. gondii*. The cyst wall of *T. gondii* tissue cysts is dissolved immediately on contact with pepsin releasing bradyzoites. Because a single tissue cyst may contain as many as 1,000 bradyzoites (Dubey et al., 1998), it is not possible to determine the density of *T. gondii* by this method. However, infection of only a small proportion of inoculated mice indicates that only a few bradyzoites were in the inoculum, perhaps released from a single tissue cyst. The results of this study indicate that the density of *T. gondii* in muscles is higher than in the brain.

Toxoplasma gondii isolates differ markedly in their virulence to outbred mice. Isolates of *T. gondii* were considered virulent when mice inoculated with tachyzoites or bradyzoites died within 28 days PI. Virulence to mice depends on several factors, including the stage of the parasite, route, dose, types of mice used, and the strain of the parasite. Among the 3 infectious stages of *T. gondii*, oocysts are more virulent than tachyzoites or bradyzoites (Dubey and Beattie, 1988). Type I isolates are more virulent to mice than types II and III. However, in this study, most isolates of *T. gondii* from cats from Paraná, Brazil, were virulent for mice, irrespective of the genotype. We recorded mouse mortality data after inoculation with feline tissues because there are no such data available from previous studies. The results concur with those from investigations with isolates of *T. gondii* from chickens from many countries, indicating that mouse virulence may not be rigidly associated with genetic type because many type III isolates from chicken from Brazil were virulent for mice (Dubey et al., 2002), whereas type I strains from chicken from Peru were not (Dubey et al., 2004). Recent studies corroborate these findings by showing that the extent of natural recombination between (unlinked) genes is sufficiently high to reduce association between alleles from different loci (Lehmann et al., 2004).

Overall, there is low genetic diversity among *T. gondii* isolates examined so far. *Toxoplasma gondii* isolates have been classified into 3 genetic types (I, II, and III) on the basis of RFLP (Howe and Sibley, 1995; Howe et al., 1997; Lehmann et al., 2000). It has been suggested that type I isolates or recombinants of types I and III are more likely to result in clinical toxoplasmosis (Fuentes et al., 2001; Grigg et al., 2001; Aspinall et al., 2003), but genetic characterization has been limited essentially to isolates from patients ill with toxoplasmosis. Unlike these reports, Ajzenberg et al. (2002) found that most (73 of 86) isolates from cases of congenital toxoplasmosis in humans from France were type II. There is very little information regarding the genetic diversity of *T. gondii* isolates circulating in

the general human population. In animals from North America and Europe, most isolates of *T. gondii* were type II or type III, irrespective of clinical status (Howe and Sibley, 1995; Mondragon et al., 1998; Owen and Trees, 1999; Jungersen et al., 2002).

Genotyping of 38 isolates obtained in this study revealed that 15 isolates were type I and 23 isolates were type III. These results are similar to those obtained from chicken isolates from Brazil (Dubey et al., 2002; Dubey, Graham, da Silva et al., 2003; Dubey, Navarro et al., 2003). Dubey, Navarro et al. (2003) surveyed chickens for *T. gondii* from the same geographic location as cats in this study. Of the 14 isolates of *T. gondii* from chickens from Paraná, 8 were type I and 6 were type III. These results indicate that type I and III genotypes are widely prevalent in this part of the world, and the *T. gondii* isolate from the cat, living near the water cistern A and presumed to be associated with the human outbreak, was not an exception. The high prevalence of *T. gondii* antibodies in cats in this study suggests that the environment in Santa Isabel do Ivai is highly contaminated with oocysts. The total absence of type II genotype isolates from cats (this study) and from chickens (Dubey et al., 2002; Dubey, Graham, da Silva et al., 2003; Dubey, Navarro et al., 2003) from Brazil is striking. Most of the isolates from animals in the United States (Dubey, Graham, Dahl, Sreekumar et al., 2003; Lehmann et al., 2003 and references contained) and Egypt (Dubey, Graham, Dahl, Hilali et al., 2003) were type II. These data support our earlier conclusion that isolates of *T. gondii* from Brazil are genetically distinct from isolates from the United States. (Lehmann et al., 2004). To our knowledge this is the first genetic characterization of *T. gondii* isolates from domestic cats.

In this study, 15 cats fed tissues infected with type I isolates shed oocysts, confirming our earlier findings that that type I isolates also circulate in nature through oocysts (Dubey et al., 2002).

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