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Short communication

Toxoplasma gondii: A study of oocyst re-shedding in domestic cats

Dauton Luiz Zulpo[a,b], Ana Sue Sammi[a], Joeleni Rosa dos Santos[a], João Pedro Sasse[a], Thais Agostinho Martins[a], Ana Flávia Minutti[a], Sérgio Tosi Cardim[a], Luiz Daniel de Barros[a], Italmar Teodorico Navarro[a], João Luis Garcia[a,*]

[a] Preventive Veterinary Medicine Departament, Universidade Estadual de Londrina, Londrina, PR, Brazil
[b] Faculty of Veterinary Medicine, Pontifícia Universidade Católica do Paraná, Toledo, PR, Brazil

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ABSTRACT

The aim of the present study was to evaluate the re-shedding of *T. gondii* oocysts in cats fed tissue cysts of homologous and heterologous strains 12, 24 and 36 months after the first infection. Thirteen cats were used in the present study and were divided into four groups: G1 (n = 2), G2 (n = 3), G3 (n = 5), and G4 (n = 3). G1, G3 and G4 cats were infected with brain cysts of ME49 and G2 with TgDoveBr8, both genotype II strains of *T. gondii*. The G1 and G2 cats were re-infected after twelve months with brain cysts of VEG strain (genotype III), and G3 cats were re-infected with TgDoveBr1 (genotype II). The G3 cats were re-infected a third time after 24 months from the second infection, and the G4 cats were re-infected 36 months after the initial infection with cysts of the VEG strain. The cats' feces were evaluated using fecal flotation and genotyped with PCR-RFLP. The serological responses for IgM, IgA and IgG were determined by ELISA. All cats shed oocysts after the initial infection. Only one G1 cat shed oocysts when re-infected after twelve months with the VEG strain. No G2 cats excreted oocysts after the second infection with VEG. G3 cats, when re-infected after twelve months with the TgDoveBr1 strain, did not shed oocysts. However, when challenged after a third time with the VEG strain, three out of four cats shed oocysts. In the G4 group, when re-infected after thirty-six months with the VEG strain, two out of three cats shed oocysts. All oocyst samples were genotyped and characterized as the same genotype from the inoculum. Protection against oocyst re-excretion occurred in 90%, 25%, and 33.4% of cats after 12, 24, and 36 months from the initial infection, respectively. Therefore, the environmental contamination by oocysts from re-infected adult cats is only 30% lower than from kittens. In conclusion, the excretion of *T. gondii* oocysts was higher in experimentally re-infected cats throughout the years, especially when a heterologous strain was used.

1. Introduction

Toxoplasma gondii is an obligatory intracellular protozoan that can infect all warm-blooded animals, birds and reptiles, including human beings (Tenter et al., 2000). However, felids are the only definitive host of *T. gondii* (Frenkel et al., 1970). Cats are considered a key component in *T. gondii* transmission, and domestic cats are important because of their direct contact with humans and because they are carnivores (Dubey, 1995). Those animals after primary infection are able to shed millions of oocysts through their feces, which contaminate the environment and, after sporulation, are infective for animals and humans (Dubey, 1995).

After the first oocyst excretion, cats usually develop immunity to the re-excretion of oocysts when challenged with homologous or heterologous strains of *T. gondii* (Dubey and Frenkel, 1974; Dubey, 1995). The long-lasting immunity to oocyst shedding in domestic cats was not known until Dubey (1995), who showed that four out of nine cats re-excreted *T. gondii* oocysts 77 months after the initial infection with tissue cysts. To our knowledge, this is the only study to investigate the long-lasting immunity in cats against *T. gondii*, and the author observed that the number of oocysts eliminated during the second infection was lower than the first.

Thus, in the present work, we describe the immunity to shedding of *T. gondii* oocysts by domestic cats infected with homologous and heterologous strains 12, 24 and 36 months after the initial infection.

2. Materials and methods

2.1. Ethics committee

The present study was approved by the Institutional Ethics Committee in Animal Use (CEUA, protocol number 51/07 and 102/12).

* Corresponding author.
E-mail address: joaoluisgarcia10@gmail.com (J.L. Garcia).

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2.2. Toxoplasma gondii strains

Four strains of *T. gondii* were used in this experiment: three genotype II strains, ME49, TgDoveBr1 and TgDoveBr8 strains (Lunde and Jacobs, 1983; Barros et al., 2014) and one genotype III strain, VEG (Dubey, 1996). Oocysts obtained from a previous study (Zulpo et al., 2012) were used to orally infect ten mice with 50 sporulated oocysts. Mice were euthanized 60 days after being infected, and their brain cysts were counted (Igarashi et al., 2008) and prepared for challenge (≅ 800 brain cysts/cat).

2.3. Cats and previous monitoring

The animals used in this experiment were selected from kittens that were abandoned at the university campus. Cats were monitored for 2 months prior to the beginning of the experiment. All cats were serum negative (titer < 16) for *T. gondii* as measured with an indirect immunofluorescence assay (Camargo, 1974), and the absence of *T. gondii* oocysts was confirmed by fecal examination.

2.4. Infection of cats

Thirteen domestic short-haired cats (*Felis catus*) of both sexes, between 3 and 6 months of age, were randomly divided into four groups: G1 (n = 2), G2 (n = 3), G3 (n = 5), and G4 (n = 3). The initial infection of the G1, G3 and G4 cats was performed with tissue cysts of ME49, and the G2 cats were infected with TgDoveBr8. One year later, the G1 and G2 cats were re-infected with VEG, and the G3 cats were re-infected with TgDoveBr1. Finally, after 36 months of the experiment, the G3 and G4 cats received another inoculum with the VEG strain (Table 1).

All infections were performed with ≅800 tissue cysts (contained in a volume of 2 mL), administered via stomach tube. After the infection, each animal was injected with 5 mL of saline. This procedure was performed with all animals anesthetized with tiletamine plus zolazepam (Zoletil, Virbac-Brazil, 3.15 mg/kg/IM). The G^1 and G^2 groups were accompanied for 15 months and the G^3 and G^4 groups were accompanied for 39 months of the experiment. During the oocyst shedding period 20 days after infections, the cats were housed in individual cages, but outside of this period, they were housed collectively in cages. The cats received only commercial feed and water ad libitum.

2.5. Oocyst shedding

Feces from each cat were collected daily from the 1st day after the challenge until the 20th day and were examined microscopically for oocysts, as described by (Garcia et al., 2007).

2.6. Enzyme-linked immunosorbent assay (ELISA) - IgM, IgA and IgG

ELISAs for IgG and IgM were performed as described by Garcia et al. (2007) while for IgA, the assay was performed as in Zulpo et al. (2012). Optimal dilutions were established by using checkerboard titrations with dilutions of sera and conjugates. Proteins from membrane tachyzoites of *T. gondii* were used as antigens (Garcia et al., 2004) to coat the flat-bottom 96-well polystyrene microtitration plates (Nunc-Immuno Plate, MaxiSorp, Denmark).

2.7. Genotyping of tissue cysts and oocysts

Samples of tissue cysts used in the inoculum and feces (a pool from each group) were used to perform the genetic characterization of *T. gondii*. Tissue cysts and feces underwent DNA extraction using a commercial kit following the manufacturer's instructions (NucleoSpin® Tissue, Macherey-Nagel, Germany). Genotyping was performed using multilocus PCR-RFLP with 11 genetic markers (SAG1, 5′-3′SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) as previously described (Su et al., 2010).

2.8. Statistical comparisons

A Wilcoxon-Mann-Whitney *U* test was used to test for significant differences in OOPG (oocysts per gram of feces) from ME49 and TgDoveBr8 at initial infection. A $p \leq 0.05$ was considered statistically significant. The prevention against oocyst elimination in cat feces was calculated by estimating the preventable fraction (PF) as previously described (Garcia et al., 2007) with modifications: PF = (P2-P1)/P2, where P2 is the total number of oocysts shed at initial infection and P1 is the total number of oocysts shed at the second or third infection.

Table 1
Oocyst shedding of cats infected with homologous and/or heterologous strain of *Toxoplasma gondii*.

Groups	Cat numbers	Age in months	Sex	Infections (months)					
				0		12		36	
				Strain (type)	OOPG	Strain (type)	OOPG/%PF[b]	Strain (type)	OOPG/%PF[b]
G1	61	5	M	ME49 (II)	14,250	VEG (III)	0/100	ND	
	65	5	M		213,250		118,750/ 44.3		
	31	6	M	TgDoveBr8 (II)	1,402,500	VEG (III)	0/100	ND	
G2	32	6	F		24,500		0/100		
	34	6	M		485,000		0/100		
	1	4	F	ME49 (II)	25,500	TgDoveBr1 (II)	0/100	VEG (III)	0/100
	2	4	F		2000		0/100		3750/nc[c]
G3	4	5	M		1,527,000		0/100		617,500/59.6
	11[a]	4	M		12,500		0/100		[a]
	23	6	M		415,000		0/100		215,000/48.2
	18	6	M	ME49 (II)	25,500	ND		VEG (III)	3750/85.3
G4	4D	3	F		304,000				0/100
	5E	3	M		3,966,500				55,000/ 98.6
Number of positive cats (%)					13(100)		1(10)		5(7)

OOPG = Total of oocyst per gram of feces during 20 days of evaluation; ND = Not done

[a] Animal 11 died by noninfectious causes before third infection (36 months).

[b] Individual preventable fraction (%) PF = (P2-P1)/P2.

[c] PF was not calculated because animal excreted more oocysts than previously.

Table 2
The dynamics of *Toxoplasma gondii* oocyst shedding by cats after infection and with homologous and heterologous strains.

Groups	infection	Number of animals	Month of infection	Strain/type	Mean pre patent period[a]	Mean patent period[a]	Mean peak of oocysts shedding[a]	Total mean oocysts shedding/cat[b]	Number of cats that excreted oocysts
G1	1st	2	0	ME49/II	3.5 ± 0.7	9 ± 2.8	7.2 ± 2.4	113,750	2
	2nd	2	12th	VEG/III	4	4	6	118,750	1
G2	1st	3	0	TgDoveBr8/II	3.7 ± 0.6	6.7 ± 2.9	5.7 ± 0.6	637,333	3
	2nd	3	12th	VEG/III	0	0	0	0	0
G3	1st	5	0	ME49/II	3.8 ± 0.4	5.4 ± 4.5	7.2 ± 2.0	660,667	5
	2nd	5	12th	TgDoveBr1/II	0	0	0	0	0
	3rd	4[c]	36th	VEG/III	5.33 ± 1.1	5.0 ± 3.0	7.7 ± 1.1	292,691	3
G4	1st	3	0	ME49/II	3.3 ± 0.5	8.6 ± 2.3	7.3 ± 1.5	1,432,000	3
	2nd	3	36th	VEG/III	6 ± 1.4	3.5 ± 0.7	7	30,843	2

[a] Period in days.
[b] Oocysts per gram of feces (OOPG).
[c] Animal number 11 died before the end of experiment.

3. Results

The overall results of oocyst shedding are shown in Table 1. All animals excreted oocysts during the primary infection with type II strains, ME49 and TgDoveBr8. There was no significant difference ($p = 0.350$) in average OOPG between ME49 (OOPG = 661,800 ± 1,247,000) and TgDoveBr8 (OOPG = 637,333 ± 701,516). After the second challenge at 12 months, the G2 and G3 cats did not shed oocysts. However, one G1 cat excreted an OOPG of 118,750, which was 44.3% fewer oocysts than in the first excretion (PF = 44.3%). Three G3 cats excreted oocysts after the third infection (36th month) with the VEG strain, with OOPGs of 3750, 617,500 and 215,000. One G3 cat (number 11) died of a noninfectious cause before the end of the experiment. The G4 cats received a second infection (VEG strain) three years after the initial one, and two of them excreted OOPGs of 3750 and 55,000.

The dynamics of *T. gondii* oocyst excretion after re-infections are shown in Table 2. The prepatent period (PPP) after primary infection with the ME49 strain (G1, G3, and G4) was 3.5 days, the patent period (PP) was 7.7 days, and peak oocyst excretion (POE) occurred on the 7.2th day. The PPP after primary infection with the TgDoveBr8 strain (G2) was 3.7 days, the PP was 6.7 days, and the POE was on the 5.7th day. The PPP, PP, and POE from the VEG strain were on days 5.1, 4.2 and 6.9. Cats inoculated with the TgDoveBr1 strain did not shed oocysts.

Cats showed IgM, IgA and IgG antibodies just after the end of the oocyst sheds of the ME49 and TgDoveBr8 strains (primary infection) and remained positive until the last analysis. There were no variations in antibody concentrations of animals after re-infection.

All fecal samples of cats (oocysts) genotyped were characterized as the same genotype used in the inoculum (tissue cysts).

Cat 11 died 31 months after the initial infection due to renal failure, which was not associated with toxoplasmosis or any infectious cause. All other cats appeared clinically normal throughout the experiment.

4. Discussion

Even 47 years after the discovery of the intestinal life cycle of *T. gondii* (Frenkel et al., 1970), little is known about long-lasting immunity in cats that have excreted oocysts (Dubey, 1995). There are some questions still to be answered, such as the following: 1) Why does this parasite perform its sexual cycle only in the enteroepithelial cells from felids? 2) How does immunity after the initial T. gondii infection influence the re-shedding of oocysts? 3) Is there any intestinal cross-immunity between different strains (genotypes)? 4) How is the humoral immune response capable of protecting against oocyst shedding? 5) Is it possible to discriminate acute and chronic infection by titering IgG, IgM, and IgA? Answering these questions is not an easy task. Herein, we tried to partially answer questions 2–5.

All animals in this study shed oocysts after the primary infections with ME49 (type II) and TgDoveBr8 (type II). Protection against oocyst re-excretion occurred in 90%, 25%, and 33.4% of cats after 12, 24, and 36 months from the initial infection, respectively. Thus, cat immunity against oocyst shedding decreased over the years. Similar results were described by Dubey (1995), when 12 cats infected with the ME49 and TS2 strains shed a high amount of oocysts in the primary infection, and after a re-infection with a homologous strain 39 days later, shed no oocysts. However, when cats were re-infected 77 months later with a heterologous strain, protection was observed in only 56% (5/9). Additionally, Davis and Dubey (1995) showed that cats that previously excreted ME49 strain oocysts did not re-shed oocysts after re-infection with the same strain three months later. Those results showed that the period between infections and homology between strains are important for intestinal immunity against *T. gondii* oocyst excretion in cats. Constantly challenged cats could be more protected than animals less exposed to *T. gondii.* Brazil has a high genetic diversity of *T. gondii* (Pena et al., 2006), thus, we could hypothesize that the intestinal immunity of cats may be related to the genotype of the strains, and the dynamics of oocyst shedding in Brazil could be different than in Europe and North America.

Cats infected with the ME49 and VEG strains can eliminate millions of oocysts on the first infection (Dubey, 1995; Garcia et al., 2007; Zulpo et al., 2012; Zulpo et al., 2017). Most cats are infected for the first time as kittens, which eliminate a smaller amount of feces than adults. An adult cat has the potential to excrete approximately 40 g of fecal matter per day while kittens approximately 10 g of fecal matter per day (Dabritz et al., 2006). Using Table 1 to calculate the average OOPG of feces from kittens at the initial shedding and multiplying by ten (grams of feces per day from a kitten), kittens have the potential to produce 6,475,000 oocysts. Meanwhile, adult cats at their last shedding (x40) will produce 4,516,000 oocysts in total. Therefore, environmental contamination by oocysts from re-infected adult cats is only 30% lower than that of kittens. The risk of infection by sporulated oocysts in human populations has been well documented (Santos et al., 2010).

According to genotyping, all oocyst samples were characterized as type II when infected with tissue cysts of ME49, TgDoveBr1 and TgDoveBr8 strains, and genotype III when cats were infected with the VEG strain. The type II strain is the most common strain associated with human toxoplasmosis in patients with AIDS, as well as in congenital infections in animals and humans, while type III is frequently associated with animal hosts (Howe and Sibley, 1995). The ME49 strain is considered to have high pathogenicity (Dubey, 1995) and is able to produce a larger amount of oocysts than the VEG strain. Furthermore, ME49 is the most frequently used *T. gondii* strain for challenging cats (Lappin et al., 1993; Dubey, 1995; Zulpo et al., 2012; Zulpo et al.,

2017). In addition, a study showed that *T. gondii* causes more severe ocular disease in congenitally infected children in Brazil compared with Europe (Gilbert et al., 2008), which demonstrates the importance of genotyping in studies on oocyst shedding in cats. This is especially true in Brazil due to the high genetic diversity of *T. gondii*.

Cats develop antibodies against *T. gondii* within 10 days of ingesting tissue cysts and animals that have antibodies are more likely to have shed oocysts before (Dubey et al., 1995). In our study, all cats seroconverted for IgM, IgA and IgG after infection with the ME49 and TgDoveBr8 strains and remain positive until the end of experiment. This lack of antibody variation did not allow discrimination between acute and chronic infection with IgG, IgM, or IgA kinetics. The high concentration of antibodies did not protect animals against the re-excretion of oocysts. Additionally, IgM in cats was detected for many months after the primary infection, showing that it does not always correlate with recent infection (Lappin et al., 1993).

Previous studies reported that antibody formation in vaccinated cats was not sufficient to induce total immunity against infection (Zulpo et al., 2012; Zulpo et al., 2017), because the main immune response against this protozoan is cellular immunity. In mice and pigs the presence of antibodies was also not sufficient for animals to become immune to *T. gondii* infection (Garcia et al., 2005; Igarashi et al., 2008).

5. Conclusion

We could assume that the quantity of oocysts shed by adult cats was lower than when they were young. However, the amount of feces that an older cat produces is much higher than a younger one. In conclusion, the excretion of *T. gondii* oocysts may stay high in experimentally re-infected cats years after first infection, especially when different strains are used, which is more likely to occur in natural conditions. The potential for environmental contamination from a cat may be much higher than previously thought because 10% and 71% of the cats shed oocysts after secondary and tertiary infections, respectively.

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