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Short communication

Investigation of *Toxoplasma gondii* in semen, testicle and epididymis tissues of primo-infected cats (*Felis catus*)

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ABSTRACT

This study aimed to investigate the presence of *Toxoplasma gondii* in semen, testicle and epididymis tissues of cats experimentally infected by this coccidium. A total of 12 male felines without a definite breed that were of reproductive age and serologically negative for *T. gondii* were selected and distributed to the following three experimental groups: GI, inoculated with 600 tissue cysts of the P strain of *T. gondii* (isolate III); GII, inoculated with 2×10^5 tachyzoites of the RH strain (isolate I); and GIII, not inoculated (control group). Prior to inoculation (day −7 and 0) and on post inoculation days (PIDs) 7, 14, 21, 28, 42, 56, and 70, all felines were subjected to assessments of anti-*T. gondii* IgG by indirect immunofluorescence (IIF) and assessments of parasitemia. Collection of semen (electroejaculation) was performed on the specified dates, followed by nested PCR and bioassays in mice to detect *T. gondii*. On PID 70, all 12 felines were orchiectomized, and the presence of the parasite in the testicles and epididymides was evaluated by nested PCR, murine bioassay, and histopathological and immunohistochemical analyses. All felines inoculated with *T. gondii* (GI and GII) seroconverted to the toxoplasmic infection after PID 14; on PID 7, seroconversion of three felines (P4, RH2 and RH4) could observed, and all exhibited detectable titers by PID 64. The GII felines exhibited greater serological titers compared with GI felines. The maximum serological titer (IgG) was observed in feline RH3 (titer 1024), while in other experimental felines, a maximum titer of 256 was detected. Parasitemic peaks were diagnosed in all felines of groups I and II from PIDs 7–42. A total of five parasitemic peaks were diagnosed in GI and nine in GII. In none of the experimental time points was the presence of *T. gondii* diagnosed in seminal samples collected from the felines or in the testicle or epididymis tissues collected from these animals. Thus, sexual transmission in domestic cats does not appear to be a major route of *T. gondii* infection, possibly demonstrating the tendency of this protozoan to develop a response directed to the formation and excretion of oocysts in the feces of these definite hosts, which act as its main route of perpetuation in the environment.

1. Introduction

Domestic felines (*Felis catus*) are the major host species responsible for the maintenance of the *Toxoplasma gondii* life cycle in the urban environment, as they are the only definite hosts of this parasite to live in these areas (Dubey et al., 2004). After becoming infected, these animals may eliminate parasite oocysts in feces, and these forms are capable of enduring different environmental stresses, which may be highly infectious for several species of mammals and birds (Dubey, 2006).

In different intermediate hosts, *T. gondii* was isolated from the semen and reproductive organs (Martinez-Garcia et al., 1996; Liu et al., 2006; Moura et al., 2007; Arantes et al., 2009; Scarpelli et al., 2009; Santana et al., 2010; Koch et al., 2016), making it possible to confirm the sexual transmission of this protozoan in dogs (Arantes et al., 2009), sheep (Lopes et al., 2013) and goats (Santana et al., 2013).

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Because of the absence of extensive research on *T. gondii* in feline semen, the present study aimed to investigate the presence of infectious forms of *T. gondii* in semen, testicle and epididymis tissues of cats primo-infected with the P (isolate III) and RH (isolate I) strains of this protozoan.

2. Material and methods

This study was performed according to the guidelines of a project previously submitted and approved by the Ethical Committee for Animal Welfare-CEUA/FCAV/UNESP, Jaboticabal, Sao Paulo, under protocol number 024137/13.

2.1. Animals

A total of 12 healthy animals (males) from the city of Jaboticabal, SP were selected. They were of no definite breed and of reproductive age (i.e., one to four years old). Serum isolated from the blood of these cats was submitted to assessments of anti-*T. gondii* antibodies (IgG) in triplicate measurements by indirect immunofluorescence (IIF) techniques; only serologically negative animals (IgG titer < 16) were selected, according to a method described by Camargo (1964).

2.2. Infective material

On day "zero" (D0), animals belonging to GI (felines P1, P2, P3, and P4) were orally infected with 600 *T. gondii* cysts (P strain type III isolate) diluted in a physiological solution. The inoculum was administered with the aid of a catheter coupled to a 10 ml syringe according to the method described by Costa et al. (1977). In GII felines (RH1, RH2, RH3 and RH4), animals were subcutaneously inoculated in the dorsal region with 2×10^5 *T. gondii* tachyzoites (RH strain type I isolate) using a 1 ml syringe and needle (0.80 mm × 30 mm; 21G). Felines belonging to GIII (C1, C2, C3 and C4) were not inoculated, and they served as a control group.

2.3. Coproparasitological examinations

Coproparasitological examinations were carried out daily using fecal samples excreted by GI felines from PIDs 1–17, in accordance with the methodology used by Dubey et al. (1972). The microscopic identification of *T. gondii* oocysts was carried out according to Costa et al. (1977) and Zaman (1970). To verify the presence of *T. gondii*'s oocysts in experimental feline feces, intraperitoneal inoculations in mice (bioassay) were also performed (Dubey et al., 1972).

2.4. Serological tests

From each feline (GI, GII and GIII), blood samples (1 ml) were collected by femoral venipuncture and were then packaged into sterile tubes (containing no anticoagulant) before inoculation (days −7 and 0) and on PIDs 7, 14, 21, 28, 42, 56 and 70. Samples were centrifuged at 1500 rpm for 10 min to obtain serum samples for IFF (Camargo, 1964).

All positive serological samples (IgG titer > 64) obtained from felines at the different experimental dates were submitted to titration of anti-*T. gondii* antibodies (IgG).

2.5. Semen collection

Semen collection in felines was carried out on days −7 and 0 before inoculation and on PIDs 7, 14, 21, 28, 42, 56 and 70.

Each semen sample was divided into two equivalent aliquots, one was stored at −20 °C and used to study *T. gondii* by polymerase chain reaction (nested PCR), and the other was immediately inoculated into mice to study the agent by murine bioassay (Lopes et al., 2009).

Table 1
Multiple comparisons among the mean serologic titer (anti-*T. gondii* IgG) obtained from the felines infected with *T. gondii* (GI and GII), on the different experimental dates.

Experimental	Experimental Groups/Mean and Standard Deviation[1]	
Dates	Group I (infected isolate III)	Group II (infected isolate I)
7	16.0 ± 32.0B	32.0 ± 37.0A
14	32.0 ± 37.0B	256.0 ± 0.0A
21	32.0 ± 37.0B	448.0 ± 384.0A
28	160.0 ± 110.9A	448.0 ± 384.0A
42	160.0 ± 110.9A	448.0 ± 384.0A
56	208.0 ± 96.0A	512.0 ± 443.4A
70	160.0 ± 110.9A	384.0 ± 554.3A

[1]Means values followed by the same letter on the same line do not differ significantly at a 95% reliability level.

2.6. Bioassay

In each semen sample aliquot, 0.5 ml of physiological solution was added; the mixture was then immediately inoculated into five mice (Costa et al., 1977).

2.7. PCR

Extraction and purification of DNA from the seminal samples were performed by using an illustra blood genomic prep mini spin kit (GE Healthcare Life Sciences do Brasil Ltda*, Brazil), according to manufacturer's instructions.

DNA samples were submitted to amplification by nested PCR using specific oligonucleotide primers to amplify a fragment of the 18S subunit of the rRNA gene as follows: Tg18s48F (5′-CCATGC ATGTCTAAGTATAAGC-3′) and Tg18s359R (5′-GTTACCCGTCACTG CCAC-3′) in the primary reaction and Tg18s58F (5′-CTAAGTATAAG CTTTTATACGGC-3′) and Tg18s348R (5′-TGCCACGGTAGTCCAATAC-3′) in the secondary reaction. These primers amplified a region of 290 base pairs (bp). As a positive control, a DNA sample extracted from tachyzoites of the *T. gondii* RH strain was used (Silva et al., 2009).

2.8. Immunohistochemistry

On PID 70, all felines (GI, GII and GIII) were orchiectomized (Lui et al., 2011) to collect testicles and epididymides.

Tissue samples (testicles and epididymides) were each divided into three aliquots so that one was used in the *T. gondii* bioassay (Costa et al., 1977), another was stored at a temperature of −20 °C and used in the parasite study by nested PCR, and the third one was fixed in 10% buffered formalin for use in histopathological and immunohistochemical analyses.

2.9. Data analysis

Comparisons among average serologic titers obtained from feline experimental groups were made using the Kruskal–Wallis test (95% confidence interval). All statistical analyses were performed using SAS software.

3. Results

On PID 7, three felines inoculated with *T. gondii* (P4, RH2 and RH4) seroconverted (anti-*T. gondii* IgG titers > 64). On PID 14, all experimental animals (GI and GII) exhibited anti-*T. gondii* IgG titers equal to 64. The maximum anti-*T. gondii* serological titer (IgG) was diagnosed in feline RH3 (titer 1024) on five experimental dates (PIDs 21, 28, 42, 56 and 70). In the other felines that belonged to groups I and II, the maximum diagnosed reciprocal serological titer was 256 (Table 1).

By coproparasitological analyses (microscopy followed by morpho-

Table 2
Number of oocysts eliminated by each feline experimentally infected with cysts from the "P" strain of *Toxoplasma gondii*.

Number of the feline	Number of oocysts excreted in 24 h/Days post-inoculation																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Total
P1	0	0	0	0	0	0	150.5	71	47	17.75	16.53	20	16.5	0	0	0	0	339.275
P2	0	0	0	0	0	0	17.11	ND	85	163.9	59.22	0	0	0	0	0	0	325.265
P3	0	0	0	0	0	33.5	88.3	124.3	1001	130	38.22	0	41	0	0	0	0	1455.77
P4	0	0	0	0	17.95	128.4	ND	ND	87.2	32.25	20.1	0	0	0	0	0	0	285.9
Total	0	0	0	0	18.0	161.9	255.9	195.3	1219.7	343.9	134.1	20.0	57.5	0	0	0	0	2406.21

ND = Not determined.

logical analyses and intraperitoneal inoculation in mice), it was possible to diagnose the presence of oocysts in the feces of all felines that had been inoculated orally with the P strain. These cats exhibited parasite tissue cysts between PIDs 5 and 13 (Table 2). All mice intraperitoneally inoculated with feline fecal samples that contained oocysts showed positive findings six weeks after inoculation, which were indicated by seroconversion (titer $>$ 32) and/or the observation of cerebral cysts.

The presence of cerebral cysts with anti-*T. gondii* serum antibodies IgG (IFF $\geq$ 32) was not diagnosed in any mice inoculated with seminal or tissue samples (testicles and epididymides) collected from the felines inoculated with *T. gondii* (GI, GII) or those in the control group (GIII).

By nested PCR, we could not detect the presence of *T. gondii* DNA in the seminal samples of male cats (GI, GII and GIII), which were collected on different post inoculation dates for the parasite. The absence of *T. gondii* DNA was also verified in tissue samples (testicles and epididymides) collected from the cats (GI, GII and GIII).

The absence of infective forms in the feline testicles and epididymides could also be verified by the histopathological and immunohistochemical analyses that we performed.

4. Discussion

By coproparasitological analyses (light microscopy and mouse bioassay) of the fecal samples collected (PIDs 1–17) from felines inoculated with tissue cysts (GI), we could verify the excretion of *T. gondii* oocysts at different levels by all animals orally inoculated with P strain tissue cysts (isolate III) of *T. gondii*. Our findings were in accordance with those of Dubey (2005), who orally inoculated young felines with parasite bradyzoites and verified the elimination of oocysts in the feces of 100% of animals. The morphological characterization and mouse bioassay performed in this present study are of extreme importance for confirming the presence of oocysts (*T. gondii*) in the feline feces, as *Hammondia hammondi and Besnoitia* spp oocysts morphologically resemble those of *T. gondii*, and it is not possible to distinguish them by light microscopy (Dubey, 2009).

According to Dubey et al. (1977), in a primo-infection, cats younger than 12 months old eliminated more oocysts than adult cats. The high amount of oocyst excretion exhibited by adult felines in the present study highlighted the importance of primo-infected cats in the dissemination of these infective forms of *T. gondii* in the environment, which is a process that is generally attributed to younger felines.

The anti-*T. gondii* IgG curve that we observed in this experiment began with a reciprocal serological titer of 64 on PID 7 (felines P4, RH2 and RH4). Navarro et al. (1998) described rapid seroconversion in cat (PID 7) carriers of experimental toxoplasmic infection. However, in contrast with the study of Navarro et al. (1998) who demonstrated a reciprocal serological maximum titer of 16,384 in felines primo-infected with the VPS strain of parasite, the maximum serological titer detected in this present study was considerably lower, reaching a maximum titer of 1024 in feline RH3 (inoculated with isolate I) from PIDs 21–70. According to Dubey (1986), antibodies related to chronic infection (IgG) can remain "active" for a long period of time, as observed in this present study, in which this class of antibodies exhibited high activity until PID 70 in felines inoculated with the RH strain (isolate I).

Antibody levels in cats after the inoculation of the parasite suggest the existence of a difference in the immunological profile among the animals in each group. These variations, which are particularly important, might be associated with the individual response capacity of each animal, as described by Sharma (1990) who attributed such differences to each host's individual capacity to resist to *T. gondii*. Furthermore, host resistance is regulated by the histocompatibility antigen H-2 genes. As the felines used in this present study were not genetically homogeneous, it seems logical that differences in individual immunological responses were observed.

In the present study, the elimination of *T. gondii* was not detected in the semen of felines inoculated with the P (isolate III) and RH (isolate I) strains of the parasite by bioassay or nested PCR on the specified experimental dates. Such findings contradict those obtained in the following studies conducted in other animal species in which the presence of *T. gondii* was diagnosed in seminal samples: humans (Martinez-Garcia et al., 1996), rabbits (Liu et al., 2006), swine (Moura et al., 2007), bovines (Scarpelli et al., 2009), canines (Arantes et al., 2009), ovines (Lopes et al., 2009) and caprines (Santana et al., 2010).

Dubey et al. (1997) inoculated cats with *T. gondii* cysts and succeed in detecting the presence of parasite in only 2 of 21 animals that were older than two months, although all oocysts in the feces were eliminated one week after infection.

Although domestic felines may manifest clinical toxoplasmosis as do other species that serve as intermediate hosts, infection caused by *T. gondii* in these animals tends to differ when compared to infections caused in intermediate hosts. This *T. gondii* behavioral difference in the infection of definite hosts could also be observed in this present study, which was in contrast to other intermediate host species because the presence of the parasite was not diagnosed in tissues or seminal samples. This difference suggested a possible tendency of the parasite to trigger infection in definite hosts by the excretion of oocysts, and further studies that use different methodologies will be needed to more rigorously test this hypothesis.

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