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Methods to produce and safely work with large numbers of *Toxoplasma gondii* oocysts and bradyzoite cysts

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Abstract

Two major obstacles to conducting studies with *Toxoplasma gondii* oocysts are the difficulty in reliably producing large numbers of this life stage and safety concerns because the oocyst is the most environmentally resistant stage of this zoonotic organism. Oocyst production requires oral infection of the definitive feline host with adequate numbers of *T. gondii* organisms to obtain unsporulated oocysts that are shed in the feces for 3-10 days after infection. Since the most successful and common mode of experimental infection of kittens with *T. gondii* is by ingestion of bradyzoite tissue cysts, the first step in successful oocyst production is to ensure a high bradyzoite tissue cyst burden in the brains of mice that can be used for the oral inoculum. We compared two methods for producing bradyzoite brain cysts in mice, by infecting them either orally or subcutaneously with oocysts. In both cases, oocysts derived from a low passage *T. gondii* Type II strain (M4) were used to infect eight-ten week-old Swiss Webster mice. First the number of bradyzoite cysts that were purified from infected mouse brains was compared. Then to evaluate the effect of the route of oocyst inoculation on tissue cyst distribution in mice, a second group of mice was infected with oocysts by one of each route and tissues were examined by histology. In separate experiments, brains from infected mice were used to infect kittens for oocyst production. Greater than 1.3 billion oocysts were isolated from the feces of two infected kittens in the first production and greater than 1.8 billion oocysts from three kittens in the second production. Our results demonstrate that oral delivery of oocysts to mice results in both higher cyst loads in the brain and greater cyst burdens in other tissues examined as compared to those of mice that received the same number of oocysts subcutaneously. The ultimate goal in producing large numbers of oocysts in kittens is to generate adequate amounts of starting material for oocyst studies. Given the potential risks of working with live oocysts in the laboratory, we also tested a method of oocyst inactivation by freeze-thaw treatment. This procedure proved to completely inactivate oocysts without evidence of significant alteration of the oocyst molecular integrity.

Keywords

Toxoplasma gondii; oocyst; bradyzoite cyst; production; purification; inactivation

1. Introduction

Toxoplasma gondii is a ubiquitous protozoal parasite capable of infecting a broad range of warm-blooded hosts, including humans (Hill et al., 2005; Tenter et al., 2000). As a result of the significant threat of oocyst exposure through waterborne routes, *T. gondii* has been classified as an NIAID Category B priority protozoan pathogen (www.niaid.nih.gov), a category shared with other major waterborne protozoan in the genera *Cryptosporidium*, *Giardia*, *Microsporidia*. The oocyst of *T. gondii* is extraordinary in its ability to survive environmental, physical and chemical insults. Common laboratory surface disinfectants (Dubey et al., 1970; Ito et al., 1975; Kucic and Wikerhauser, 1996; Wainwright et al., 2007b) as well as the physical and chemical methods used to treat drinking and sewage water (Wainwright et al., 2007a; Wainwright et al., 2007b) do not reliably inactivate all oocysts. Furthermore, oocysts can persist and remain infective in the environment for years (Dumetre et al., 2008; Lindsay and Dubey, 2009).

Despite the importance of the oocyst stage of *T. gondii* as a major source of infection, studies that focus on its transmission, environmental distribution, resistance to inactivation and composition are extremely limited (Elmore et al., 2010; Jones and Dubey). We believe that this is largely a result of the difficulty in reliably producing large numbers of oocysts and safety concerns pertaining to working with this highly infectious and environmentally resistant organism. Oocyst production is an intensive and expensive endeavor with an inherent risk of either obtaining too few oocysts or no oocysts at all. In our past experience, oocyst shedding in kittens following infection with bradyzoite cysts in mouse brains was unreliable.

Toxoplasma gondii is an intensely studied organism and serves as a model apicomplexan for studies involving immunology, host-parasite interactions, and genetics. The vast majority of these studies utilize the bradyzoite and tachyzoite life stages, which are comparatively easy to work with and manipulate as they can be maintained and expanded *in vitro*, and are routinely inactivated with standard laboratory disinfection reagents, such as bleach and alcohol (Kim and Weiss, 2004). Like oocyst studies, *in vivo* bradyzoite studies are also limited because of the difficulty in producing sufficient numbers of bradyzoite cysts in mice and purifying them from host cells. Bradyzoite cysts generated and purified by the methods described in this paper were also used to obtain sufficient RNA for the first transcriptomic study of *in vivo* bradyzoites (Buchholz et al., in press).

Various methods of mouse inoculation have been used to produce bradyzoite cysts, including: peroral (PO) inoculation of oocysts (Arkush et al., 2003); subcutaneous (SQ) inoculation of oocysts (Arkush et al., 2003; Dubey, 2006; Radke et al., 2003); subcutaneous inoculation of bradyzoite cysts or brain homogenate containing cysts (Dubey, 2006); and intraperitoneal (IP) inoculation of culture-derived tachyzoites (Shapiro et al., 2009). Previous studies have suggested that compared to oral inoculations, the subcutaneous route of inoculation using either oocysts or bradyzoites (free bradyzoites or cysts in tissue homogenates) may be the most reliable method for mouse infection (Arkush et al., 2003; Dubey, 2006).

The aim of this paper is to share methods that have proven successful in our laboratory to generate large numbers of bradyzoite cysts in mouse brains and oocysts in cats. A comparison between oral and subcutaneous inoculation of oocysts in mice is presented as well as a method to inactivate oocysts for safe handling in downstream applications. An optimized method for harvesting bradyzoite cysts from mouse brains is also provided in the Materials and Methods.

2. Materials and Methods

All animal experiments were conducted with the approval and oversight of the Institutional Animal Care and Use Committee at the University of California Davis, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, (IACUC # 15619).

2.1 Parasite strain and infection of mice

Initial production of *Toxoplasma gondii* oocysts used to infect mice in the following experiments were produced in kittens in our laboratory using methods similar to those previously described (Wainwright et al., 2007b). The oocysts were from a type II isolate of *T. gondii* (M4) donated to our laboratory by the Moredun Research Institute, Edinburgh, Scotland. (Gutierrez et al., 2010). This strain was genetically characterized at multiple polymorphic gene sites and sequences were identical to type II ME49 at each site by ToxoDB BLAST analysis (data not shown).

2.2 Group one - mouse infection and purification of bradyzoite cysts

To evaluate numbers of cysts recovered from the brains of mice inoculated with *T. gondii* oocysts by each infection route, SQ versus PO, two groups of 4, 8-10 week-old female Swiss Webster (SW) mice, (Charles River Laboratories, Wilmington, MA), were infected by each route. The first group received 1,000 oocysts SQ in 200 µl phosphate buffered saline (PBS) delivered with a 1 ml syringe and a 25-gauge needle under the skin in the interscapular region. The second group received 1,000 oocysts in 200 µl Clinicare Canine/Feline Liquid Diet (Abbott Health Care) PO by gastric gavage. Both oocyst preparations were from the same starting oocyst preparation that was originally spiked into the delivery suspension medium (PBS or Clinicare). To minimize morbidity and prevent death in infected mice, all infected mice were treated with sulfadiazene (0.4 µg/ml in drinking water) for 10 days, beginning 10 days post-infection. Twenty-one days post-inoculation (dpi) mice were humanely euthanized by CO₂ asphyxiation. Brains were removed from each mouse, put into a 1.5 ml microcentrifuge tube in 1 ml sterile PBS and stored at 4°C for further processing that same day. One quarter of each brain was reserved for histological examination. The methods used to harvest bradyzoite cysts from mouse brains were modified from previously described protocols (Blewett et al., 1983; Huskinson-Mark et al., 1991). Three-quarters of each brain was passed through a 100 µm cell strainer into a 50 ml conical tube using the plunger of a 6 ml syringe to press the tissue through the strainer (retaining the fatty tissue in the strainer) and washed with PBS to a total volume of 4 ml. The brain suspension was then syringe-passed through a 16 gauge blunt needle 10 times followed by a 22 gauge blunt needle 10 times. The first individual cyst counts were made on

the 4 ml suspensions. The brain suspensions were then brought up to a total volume of 10 ml with PBS. A density gradient was prepared for each sample by carefully layering (from bottom to top) 9 ml 90% Percoll, followed by 9 ml 30% Percoll, and then followed by 10 ml brain suspension in a 50 ml conical tube. Percoll dilutions were made using 1X PBS. Gradient preparations were centrifuged at 1,200 x g for 15 minutes at 4°C. The cysts were harvested from the 30% and 30%/90% interface. Cyst suspensions were washed with PBS by bringing the volume up to 45 ml with PBS and centrifuging at 1,500 x g for 15 min at 4°C. The supernatant was removed to about 5 ml and the pellets were combined into one 50 ml tube. The 'first pooled' cyst count was taken at this stage. A second wash in PBS was performed by bringing the combined suspension up to 45 ml with PBS and centrifuging at 2,500 x g for 15 minutes at 4°C. The supernatant was removed and the remaining pellet was transferred to a 1.5 ml microcentrifuge tube and brought up to 1 ml with PBS. A 10 µl aliquot was removed for the 'final pooled' cyst enumeration. All cyst counts were performed by aliquoting 10 µl of cyst suspension onto a glass slide and applying a coverslip before examination by light microscopy. The entire area under the coverslip was counted and a total estimated cyst number was calculated.

2.3 Group 2 - infection of mice and tissue preparation to evaluate the tissue cyst distribution in SQ vs PO inoculated mice

To evaluate potential differences in bradyzoite cyst numbers and distribution in the tissues of mice inoculated by each route, 2 groups of 8-10 week-old female SW mice (Charles River Laboratories, Wilmington, MA) were inoculated with 1,000 oocysts: subcutaneously (n=12) and orally by gastric gavage (n=12). Control mice were given inocula free of oocysts (n=6). SQ inocula were delivered in a 200 µl PBS suspension and PO inocula were prepared in a 200 µl Clinicare suspension, as described for group one mice. Seven days following inoculation mice were treated with sulfadiazene (0.4 µg/ml) for 10 days in their drinking water. Mice were euthanized 28 dpi by CO₂ asphyxiation and tissues were prepared for histology as follows. The brain was removed from the cranium immediately after euthanasia and stored in a tissue cassette in 10% formalin. The abdominal and thoracic cavities were opened and the entire carcass was submerged in 10% formalin. Tissues were formalin-fixed for 24 hours before trimming for paraffin embedding. Boney sections were further fixed and decalcified in Surgipath Decalcifier I (Surgipath Medical Industries) for a subsequent 24 hours prior to trimming and paraffin embedding. Trimming of tissues from each mouse was done as uniformly as possible, taking care to obtain a distribution of sections from each tissue collected. For example, one half of brain was cross sectioned into six segments including 4 cross sections of cerebrum and two through the caudal brainstem that included cerebellum. There was some variation in the volume of caudal brainstem and cerebellum dependent on brain harvesting from cranium where it was sometimes difficult to remove these structures without damaging the tissue. The following tissues were examined for bradyzoite cysts by immunohistochemistry: brain, heart, muscle, lung, eye, cervical spinal cord, thoracic spinal cord and lumbar spinal cord.

2.4 Group 3 - Infection of mice for oocyst production in kittens

Twenty 8-10 week-old female SW mice (Charles River Laboratories, Wilmington, MA) were inoculated SQ with 1,000 oocysts suspended in 200 µl PBS. In addition to the SQ

oocyst inoculation, two of the mice were also inoculated PO with 1,000 oocysts suspended in 200 µl Clinicare and one mouse was additionally inoculated intraperitoneally (IP) with brain homogenate from a previously infected mouse suspended in a 200 µl PBS. The additional PO and IP inoculations were made because at that time we did not know the reliability of infection following SQ inoculations, whereas we were familiar with the outcomes from the other routes. Mice were bled every two weeks (beginning 3 weeks after inoculation), and the indirect fluorescent antibody test (IFAT) was used as described (Wainwright 2007a) to monitor *T. gondii* seroconversion. Eight weeks after infection, 10 mice were sacrificed and their brains collected. Half of each brain was fed to two twelve-week-old kittens. The other half was submitted for histology to verify the presence of bradyzoite cysts in the brain.

2.5 Kitten Infection

Two 12-week-old specific-pathogen-free kittens (Nutrition and Pet Care Center, Department of Molecular Biosciences, University of California, Davis) were used for oocyst production. Prior to infection, kittens were screened as described (Dabritz et al., 2007) using an IFAT at 1:40 dilution to ensure that they were seronegative for *T. gondii* antibodies. Additionally, feces were collected daily and examined for intestinal parasites, including protozoa. Kittens were fed a total of 2.5 mouse brains each. One kitten received brains from group A mice and the other was fed brains from group B mice (Table 3).

A second oocyst production was performed using methods similar to those described here, with a few exceptions: all mice used were inoculated either SQ or PO with 1,000 oocysts (ie. no IP brain homogenate or combination of SQ and PO routes of infection); three kittens were used; and each kitten received a pooled total of 3 brains each (1-1.5 brain equivalents were from SQ-inoculated mice and 1.5 - 2 brain equivalents were from PO-inoculated mice), instead of 2.5 brains. A combined total of greater than 1.8 billion oocysts were harvested from the second group of kittens over a 4-day collection period. Oocyst harvest from feces in the second production was discontinued after the peak days of shedding (data not shown).

2.6 Oocyst Harvest from Kitten Feces

Feces were collected from kittens daily and examined by zinc sulfate double centrifugation to detect shedding of *T. gondii* oocysts as well as to monitor for co-infection with other parasites. Once *T. gondii* oocysts were detected in feces, all procedures were conducted in a biohazard hood and unsporulated oocysts were harvested from feces using sodium chloride (sg 1.20) to concentrate the oocysts by flotation (Wainwright et al., 2007b). Oocysts were enumerated on a hemocytometer.

2.7 Statistical Analysis

A two-sample Wilcoxon rank-sum (Mann-Whitney) test was used to compare the number of cysts recovered from brain homogenates in the SQ- and PO-inoculated groups. A Fisher's exact test was used to compare tissue distribution in SQ-versus PO-inoculated mice with individual comparisons made with each tissue type and a comparison of total numbers of positive tissues between groups. The limit of statistical significance of the tests was defined as $p = 0.05$.

2.8 Oocyst inactivation for downstream applications

Oocysts were suspended in PBS (15,000 oocysts in 600 µl PBS) in a 1.5 ml screw-top microcentrifuge tube and submerged in a dry ice-ethanol bath for two minutes and then thawed in a heated block water bath at 27°C for two minutes. This was repeated for a total of three freeze-thaw cycles. Loss of infectivity was tested by bioassay: three mice were inoculated SQ with 5,000 freeze-thaw treated oocysts and one control mouse received 1,000 untreated oocysts SQ to verify oocyst infectivity prior to freeze-thaws. Mice were humanely euthanized by CO₂ asphyxiation 6 weeks post-inoculation.

To evaluate infection status of each animal, blood was collected for IFAT and DNA was extracted from brain, heart and tongue. DNA from each tissue was extracted in triplicate and nested PCR amplification of the B1 gene was performed as described (Grigg and Boothroyd, 2001) using the following primer pairs: Outer -Pml/S1, 5'-TGTTCTGTCCTATCGCAACG-3' and Pml/S2, 5'-TCTTCCCAGACGTGGATTTC-3', and inner - Pml/AS1, 5'-ACGGATGCAGTTCCTTTCTG-3' and Pml/AS2, 5'-CTCGACAATACGCTGCTTGA-3'.

3. Results

3.1 Bradyzoite recoveries from mice infected SQ versus PO

The total number of bradyzoite cysts harvested from the 4 mice that each received 1,000 oocysts inoculated SQ was 1,200 cysts in the final pooled count with cyst counts from individual mice ranging from 0-1,200. The total number of bradyzoite cysts harvested from similar brain samples from 4 mice inoculated with 1,000 oocysts PO was 48,900 in the final pooled count with cyst counts from individual mice ranging from 8,100-67,500. The bradyzoite cyst recovery was significantly higher in the PO-inoculated group as shown in Table 1.

3.2 Parasite tissue distribution in mice inoculated SQ vs PO

In a separate experiment the distribution of bradyzoite cysts in the tissues of mice that were infected with oocysts either by the SQ or PO route was evaluated. The results, shown in Table 2, are reported as either positive or negative for bradyzoite cysts in each tissue type examined. In the comparison of mice inoculated SQ versus PO, the difference in the number of positive tissues for each tissue type was not statistically significant. Four mice inoculated PO became morbidly ill (mice 9-12) and were excluded from the analysis, but included in the table. All control mice were negative for parasites in all tissues examined and were not included in the tables. A comparison of the total number of positive tissues across all tissues examined for each inoculation group showed that there were more cysts in the PO inoculated group of mice than in the SQ inoculated mice, with cysts present in 18/96 (18.8%) of tissues examined in the SQ-inoculated group compared to 24/64 (37.5%) in the PO-inoculated group.

3.3 Kitten Infections

Two kittens infected with *T. gondii* by feeding brains of mice that were infected with oocysts (Table 3) shed a combined total of approximately 1.3 billion oocysts (Fig 1). Oocyst

shedding began 4 days after feeding mouse brains. There was a sharp peak in the number of oocysts recovered from feces on days 2 and 3 of shedding before the recoveries dropped. By day 13, very few oocysts were detected at which time harvest and enumeration were discontinued (data not shown).

3.4 Oocyst inactivation

The efficacy of oocyst inactivation by the ethanol/dry-ice freeze thaw method was assessed by bioassay in mice. All mice that received 5,000 freeze-thaw treated oocysts remained seronegative at <1:40 by IFAT whereas the mouse that received 1,000 untreated oocysts had a titer >1:1280 (Table 4). None of the tissues tested in the freeze/thaw group were positive by PCR (n=27); however, 6 out of 9 tissues tested positive by PCR in the control mouse (Table 4).

4. Discussion

This study provides a comparison of two methods, subcutaneous and oral inoculation, for infecting mice with *Toxoplasma gondii* oocysts; both of which resulted in bradyzoite cysts in the brains of experimentally infected mice. The methods described resulted in the production and purification of large numbers of *in vivo* bradyzoite cysts, large numbers of oocysts and a reliable method to inactivate oocysts for downstream applications. Detailed description of procedures to accomplish these goals will be valuable to other researchers interested in conducting studies that require large numbers of oocysts and/or *in vivo*-derived bradyzoite cysts.

Both methods of inoculation evaluated in these studies have advantages and disadvantages. The oral route of oocyst infection in mice yielded significantly greater numbers of brain and tissue cysts than in mice inoculated subcutaneously. One disadvantage of oral inoculation was the increased morbidity in PO-infected mice, with 4 of the 12 orally inoculated mice becoming morbidly ill despite treatment. Two of these mice died acutely and the other 2 were euthanized. All four mice had histological evidence of severe disseminated toxoplasmosis. By comparison, all SQ-inoculated mice in this study remained asymptomatic. While resulting in a lower cyst load, there are several distinct advantages to SQ inoculations of oocysts in mice. SQ inoculations require less technical skill than oral gavage or intraperitoneal injections. There is reduced risk of environmental contamination with oocysts when delivered subcutaneously as compared to oral gavage, where dripping from the gavage needle may occur and oocysts that pass through the gastrointestinal tract may pass in the feces of inoculated animals. SQ inoculations require minimal restraint and handling of mice, which minimizes stress to mice. Unlike intraperitoneal injections, which can cause septic peritonitis in mice if the intestines are inadvertently punctured, SQ injection-related injury is negligible. Lastly, there is significantly less acute morbidity and mortality in mice that are inoculated SQ compared to PO.

Dubey (2006) compared the infectivity of oocysts delivered orally versus subcutaneously to mice and showed that the subcutaneous route more reliably resulted in infection; however, the number and distribution of cysts in the tissues was not reported (Dubey, 2006). It has been speculated that the reason for inconsistent success of infections with oocysts delivered

orally may be a result of rapid gastrointestinal (GI) transit, such that the oocysts pass through without time for sporozoite excystation and enterocyte invasion (Arkush et al., 2003). To address this possibility, we utilized a method of suspending oocysts for oral inoculations in a calorie-dense liquid replacement diet, to slow GI transit time. This method resulted in 100% infection rates in mice inoculated PO with as few as 1 oocyst (Rejmanek et al., 2010).

In our study, mouse brains fed to two kittens resulted in the production of more than 1.3 billion oocysts that were shed in their feces from days 4-12 post-infection. A second oocyst production in three kittens resulted in the recovery of greater than 1.8 billion oocysts from the feces of three kittens in the 3 peak days of shedding. Because oocysts were only harvested for the 3 peak days of shedding, the total numbers of oocysts shed and duration of shedding was not determined for the second group of kittens.

It is suspected that the use of the oocyst, instead of bradyzoites or tachyzoites for mouse infection, was instrumental to the production of the high levels of bradyzoite cyst formation in brains of mice in our study. The *T. gondii* isolate we used was originally obtained from an aborted sheep fetus (Gutierrez et al., 2010) and has been maintained in our laboratory via oocyst to mouse and bradyzoite cyst to cat. Therefore the M4 isolate used in this study has had little opportunity to become lab-adapted by serial passage *in vitro*. The infection of mice with oocysts rather than bradyzoites or tachyzoites is also considered a more natural inoculum for mice, considering that these animals are more likely to acquire their horizontal infections from oocyst contaminated environments than from carnivorousism (Dubey, 2001, 2006; Tenter et al., 2000).

In addition to comparing methods of cyst and oocyst production, a method of inactivating oocysts for safe handling in experimental applications was evaluated. Due to their resistance to most methods of inactivation, oocysts are often subjected to harsh methods of inactivation (such as boiling or high doses of UV radiation), which may disrupt the molecular integrity of oocyst properties and structures for downstream experimental applications (Dumetre et al., 2008; Shapiro et al.). Oocysts that were inactivated by the freeze-thaw method described were subsequently utilized in experiments where they were analyzed by HPLC and mass spectrometry, with good results (data not shown here, manuscript in preparation).

5. Conclusions

Both subcutaneous and oral inoculations of *T. gondii* oocysts in Swiss Webster mice resulted in bradyzoite tissue cysts in the brains of infected mice, which in turn resulted in shedding of large numbers of oocysts by kittens fed infected mouse brains. Oral inoculations with oocysts produced significantly more bradyzoite cysts in the brain and a greater overall distribution of cysts in other tissues tested as compared to the subcutaneous inoculation method. The major advantages to oral inoculations include greater parasite burdens in mouse tissues and emulation of a more natural route of infection. The work presented here is expected to be of significant value to the scientific community in that it reports detailed methods to reliably purify large numbers of *in vivo* bradyzoite cysts from the brains of mice, produce large numbers of oocysts in cats and an oocyst inactivation method to safely work

with oocyst materials. Work on the oocyst stage is critical to the advancement of our understanding of its role in human and animal infections and will direct strategies to control toxoplasmosis resulting from oocyst infections.

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Figure 1.

Total numbers of *Toxoplasma gondii* oocysts harvested from feces pooled from two kittens (first production) and three kittens (second production). Days 1-5 of oocyst shedding are shown for production 1. Days 1-4 of oocyst shedding are shown for production 2, after which time oocyst collection was discontinued. In both productions Day 1 of shedding began 4 days post-infection.

Table 1

Comparison of bradyzoite cyst counts from brain samples of mice inoculated subcutaneously (SQ) versus orally (PO) with *Toxoplasma gondii* oocysts (n=4 mice per route).

SQ inoculation of oocysts		PO inoculation of oocysts	
Mouse #	Cyst Counts ^a	Mouse #	Cyst Counts ^a
1	1,200	1	8,100
2	800	2	26,400
3	0	3	23,450
4	400	4	67,500
Total	2,400	Total	125,450
Mean # cysts per mouse	600	Mean # cysts per mouse	31,362.5
Pooled count after 2x wash	1,200	Pooled count after 2x wash	48,900

Two-sample Wilcoxon rank-sum test of differences in cyst counts obtained from each mouse in the two groups (SQ-inoculated and PO-inoculated), statistically significant (p=0.014).

^a Sample from ¾ of each brain.

Bradyzoite cysts detected by immunohistochemistry in tissues taken from mice experimentally infected with *Toxoplasma gondii* oocysts by subcutaneous or oral inoculation.

Table 2

2a. Subcutaneously inoculated mice										
Mouse #	Brain	Heart	Muscle	Lung	Eye	Spinal Cord Regions ^a				
	(+/-)	(+/-)	(+/-)	(+/-)	(+/-)	Cervical	Thoracic	Lumbar	(+/-)	
1	+	-	+	-	-	+	+	-	-	
2	-	-	-	-	-	-	-	-	-	
3	-	-	-	-	-	-	-	-	-	
4	+	-	+	-	-	-	+	-	-	
5	+	-	-	-	-	-	-	-	-	
6	+	-	+	-	-	-	+	-	-	
7	+	-	-	-	-	-	+	-	-	
8	-	-	+	-	-	-	-	-	-	
9	+	-	-	-	-	-	-	-	-	
10	+	-	-	-	-	-	-	-	-	
11	+	-	-	-	-	-	-	-	-	
12	+	-	-	-	-	-	-	-	-	
Total positive:	9/12 (75%)	0/12 (0%)	4/12 (33%)	0/12 (0%)	0/12 (0%)	1/12 (8%)	4/12 (33%)	0/12 (0%)		

2b. Orally inoculated mice									
Mouse #	Brain	Heart	Muscle	Lung	Eyes	Spinal Cord Regions ^a			
	(+/-)	(+/-)	(+/-)	(+/-)	(+/-)	Cervical	Thoracic	Lumbar	(+/-)
1	+	-	+	+	-	+	+	-	-
2	+	-	+	-	-	-	-	-	-
3	+	-	+	-	-	-	-	-	-
4	+	-	-	-	-	+	-	-	-
5	+	-	+	-	-	+	+	+	+
6	+	-	-	-	-	-	-	-	-
7	+	-	+	+	-	+	+	+	+
8	+	-	-	-	-	-	-	-	-
9 ^b	-	-	-	-	-	-	-	-	-
10 ^b	-	-	-	-	-	-	-	-	-
11 ^c	+	-	+	-	-	+	+	+	+
12 ^c	+	-	+	-	n/o [*]	-	+	+	+
Total positive:	8/8 (100%)	0/8 (0%)	5/8 (63%)	2/8(25%)	0/8 (0%)	4/8 (50%)	3/8 (38%)	2/8 (25%)	

Comparison between SQ and PO groups showed a statistically significant difference between the groups in the total number of positive tissues of all types (p=0.010 using a two-sided Fisher's exact test).

^a Spinal cord was examined across three sections: cervical, thoracic and lumbar.

^b Died 14 days PI.

^c Euthanized 16 days PI.

^{*} n/o = not obtained.

Shaded area indicates samples not included in the statistical analysis because the mice died or were euthanized prior to completion of study.

Table 3

Infection dose and route, brain cyst numbers and serologic titers for mice used to infect kittens for *Toxoplasma gondii* oocyst production

Mouse # (group)	Oocyst dose and route of infection	Cysts per histology section ^a	Serologic titer at necropsy ^b
1(A)	1,000 SQ	31-39	40,960
2(A)	1,000 SQ	0-3	20,480
3(A)	1,000 SQ	0-3	5,120
4(A)	1,000 SQ + 1,000 PO	9-19	10,240
5(A)	1,000 oocysts SQ	0-1	2,560
1(B)	1,000 SQ + 1,000 PO	>50	40,960
2(B)	1,000 SQ + cysts IP ^c	5-9	20,480
3(B)	1,000 SQ	1-4	5,120
4(B)	1,000 oocysts SQ	2-18	20,480
5(B)	1,000 oocysts SQ	0-2	2,560

Two kittens were infected by feeding the brains of the mice. One of the kittens received ½ of each brain from the group A mice and the other kitten received ½ of each brain from the group B mice.

^a Half of each brain was examined by immunohistochemistry (see materials and methods). The range of cysts numbers observed across all sections examined is reported.

^b Titer is inverse dilution of number shown.

^c Intraperitoneal (IP) inoculation of brain homogenate containing bradyzoite cysts.

Table 4
Amplification of *Toxoplasma gondii* DNA by PCR from tissues of mice inoculated with freeze-thaw or untreated oocysts

Mouse #	Oocyst treatment F/T or U/T ^a	PCR positive tissues (n/3)			Serologic titer at necropsy ^b
		Brain	Tongue	Heart	
1	5,000 F/T oocysts SQ	0/3	0/3	0/3	<40
2	5,000 F/T oocysts SQ	0/3	0/3	0/3	<40
3	5,000 F/T oocysts SQ	0/3	0/3	0/3	<40
4	1,000 N/T oocysts SQ	3/3	1/3	2/3	>1,280

^a F/T is freeze-thaw treated in dry-ice/ethanol as described in materials and methods and U/T is untreated control oocysts from same oocyst stock.

^b Titer is inverse dilution of number shown.