

RESEARCH NOTE

OCCURRENCE AND GENETIC CHARACTERIZATION OF *GRA6* AND *SAG2* FROM *TOXOPLASMA GONDII* OOCYSTS IN CAT FECES, KUNMING, CHINA

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Abstract. *Toxoplasma gondii*-like oocysts were detected in 4% of cat feces using light microscopy in Kunming, China. Amplicon was generated from these oocysts using a *T. gondii*- but not *Hammondia hammondi*-specific primer set. Cat and KM mouse were susceptible to infection by these *T. gondii*-like oocysts. Tissue cysts were found in the brain of the experimentally infected animals and were infective to mouse, indicating that the *T. gondii*-like oocysts isolated from naturally infected cats had a facultative secondary host life cycle. Comparison of *GRA6* and *SAG2* sequences with those of reference strains indicated that the cat-derived *T. gondii* (KM isolate) were typical of *T. gondii* genotype II. This is the first report of isolation, identification, and genotyping of *T. gondii* from feline feces in China. The occurrence of oocyst shedding in the cat population studied was higher than that found in most cat populations world-wide, which strongly implies the need for further studies of population genetic structure of *T. gondii*, as well as for prevention and control of *T. gondii* infection in cats in China.

Keywords: *Toxoplasma gondii*, cat, fecal oocyst, genetic characterization

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite, which infects a wide range of warm-blooded vertebrates. Human infections are primarily caused by ingesting uncooked or insufficiently cooked flesh that contains viable *T. gondii* oocysts or ingesting food

or water contaminated with oocysts shed in feces of infected felids (Dubey, 2004). Cats are the only domestic animals, which serve as definitive hosts of *T. gondii* and thus play a key role in the epidemiology of toxoplasmosis.

Little is known regarding the prevalence of cats shedding *T. gondii* oocysts in China. One of the reasons for the absence of accurate information concerning *T. gondii* is the presence of *Hammondia hammondi*, which also employs cats as definitive hosts. Moreover, the two species are

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morphologically very similar, so that differentiation depends mainly on molecular techniques. Four DNA sequences, namely, B1 gene, 529 bp element, internal transcribed spacer (ITS-1) sequence, and 18S rDNA sequence, are often used for detection of *T. gondii* in biological samples, with the 529 bp element being a more sensitive PCR target for *T. gondii* detection and will probably be more generally used (Montoya and Liesenfeld, 2004; Su *et al*, 2010). Two *H. hammondi*-specific primer pairs, CT1/HhamITS and Hham34F/Hham3R, based on ITS-1 region and a repetitive DNA fragment, respectively, have been developed for specific differentiation of *H. hammondi* from *T. gondii*, or other related cyst-forming coccidians (Sreekumar *et al*, 2005; Schares *et al*, 2008b).

The genetic diversity of *T. gondii* in various geographical regions has been extensively investigated (Dubey, 2010). A variety of different methods exist to genotype *T. gondii* isolates, and each technique has its own distinct advantages and disadvantages. Sequence-based methods have been employed for detecting polymorphism in new isolates or from a previously unexamined population (Sibley *et al*, 2009). Loci used for sequence-based analysis include coding regions of housekeeping genes and antigens, and selectively neutral introns. The coding regions of *GRA6* and *SAG2*, encoding an organelle antigen and one of several tachyzoite surface antigens respectively, are key targets as their sequences are more variable than other *T. gondii* coding genes examined so far, and should be good markers for strain characterization and typing of *T. gondii* isolates (Fazaeli *et al*, 2000; Fazaeli and Ebrahimzadeh, 2007).

The present study employed morphological data, experimental infections, and molecular techniques to examine cat

feces for the presence of *T. gondii*. In addition, *T. gondii* isolated from cat feces were genotyped based on DNA polymorphisms of *SAG2* and *GRA6* loci.

MATERIALS AND METHODS

Samples collection

A total of 115 fresh cat feces were collected during March 2010 to November 2011 from a refuge facility in Kunming City, the capital of Yunnan Province, located in southwest mainland of China. The history of these animals was unclear. All fecal samples were examined using a flotation method with a saturated salt solution (specific gravity of 1.20) (Zuo, 1992). Surface material was observed using light microscopy (200x magnification) and oocysts, 9-14 μm in diameter and exhibiting morphology similar to those of *T. gondii*, were used for *in vitro* sporulation, subsequent PCR identification, and experimental infection.

PCR identification of putative *T. gondii* oocysts

Genomic DNA from approximately 50 putative *T. gondii* oocysts isolated from each infected cat was extracted using phenol/chloroform method after 5 rounds of freezing and thawing, and digestion with 0.01% proteinase K and 0.25% trypsin. For differentiating between *T. gondii* and *H. hammondi*, PCR was performed with *T. gondii*-specific primers Tox4/Tox5 (Homan *et al*, 2000) and 2 sets of *H. hammondi*-specific primers, CT1/HhamITS (Sreekumar *et al*, 2005) and Hham34F/Hham3R (Schaes *et al*, 2008b). PCR was performed in a total volume of 25- μl PCR cocktail that included 1X PCR buffer, 0.15 mmol MgCl_2 , 0.25 mmol dNTPs, 1 U *Taq* DNA polymerase (TakaRa, Dalian, China), 50-100 ng of DNA, and 25 pmol of each primer. Thermocycling was performed in

a TProfessional Thermocycler (Biometra, Gottingen, Germany) as follows: 35 cycles of 95°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute. Gel-purified amplicons were sequenced using an ABI 3730 XL automatic sequencer (Applied Biosystems, Carlsbad, CA) using the same PCR forward and reverse primers. Sequence was assembled from multiple overlapping regions using Seqman II program (DNA-Star, Madison, WI) and was deposited in GenBank (accession no. KX781159).

Experimental infection

Eight, 1- to 2-month-old domestic cats were housed separately in steel cages and fed cat chow and water *ad libitum*. Cat feces were examined for 15 successive days using flotation method to confirm they were free of coccidians. KM (Kunming) mice (*Mus musculus*) obtained from the Experimental Animal Center, Kunming Medical University, Kunming, China were housed in groups of 2 per plastic cage and were provided rodent chow and water *ad libitum*.

Three separate experiments were performed as follows:

Experiment 1: Test of *T. gondii* oocysts infectivity of definitive and intermediate hosts. Approximately 200 sporulated *T. gondii* oocysts were fed to 1 cat and 2 KM mice, with the same number of animals used as uninfected controls. Fecal samples were examined daily for 60 days post-infection (PI) to determine the presence of *T. gondii* oocysts. All animals were euthanized after 60 days PI and brain smears were examined for *T. gondii* cysts using a light microscope.

Experiment 2: Test of *T. gondii* cyst infectivity of definitive host. Two cats were fed on the carcasses of infected animals from Experiment 1 and 2 cats were fed on carcasses of control animals (a single

carcass for each animal). Feces of all cats were examined daily for 20 days for *T. gondii* oocysts shedding.

Experiment 3: Test of *T. gondii* cyst infectivity of intermediate host. Two KM mice were fed with carcasses of infected KM mice from Experiment 1 and 2 KM mice served as controls. All mice were euthanized after 60 days PI and brain smears were examined for *T. gondii* cysts by light and transmission electron microscopy. In the latter, brain tissues containing *T. gondii* cysts were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 at 4°C and post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in graded alcohol solutions, and embedded in epon-araldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using JEM100-CX transmission electron microscopy (Jeol, Tokyo, Japan).

Genotype analysis of *T. gondii* KM isolates

One cat was fed on carcasses of 2 infected KM mice and another serving as a negative control was fed on carcasses of 2 control KM mice. All cat feces were examined for *T. gondii* oocysts for 20 days. Oocysts were collected after sporulation and stored at 4°C for genotype analysis. Coding region of *GRA6* and *SAG2* was amplified using primer pairs, Forward: GTAGCGTGCTTGTTGGCGAC and Reverse: TACAAGACATAGAGT-GCCCC (Fazaeli *et al*, 2000), and Forward: GAAATGTTTCAGGTTGCTGC and Reverse: AAGGTTTCACGAAGGCACAC (Howe and Sibley, 1995), respectively. Genomic DNA extraction and PCR amplification were performed as described above. Gel-purified amplicons were cloned into PMD-19T vector (TakaRa). Three clones of each PCR product were sequenced, and then the sequences were assembled using the SeqMan II program.

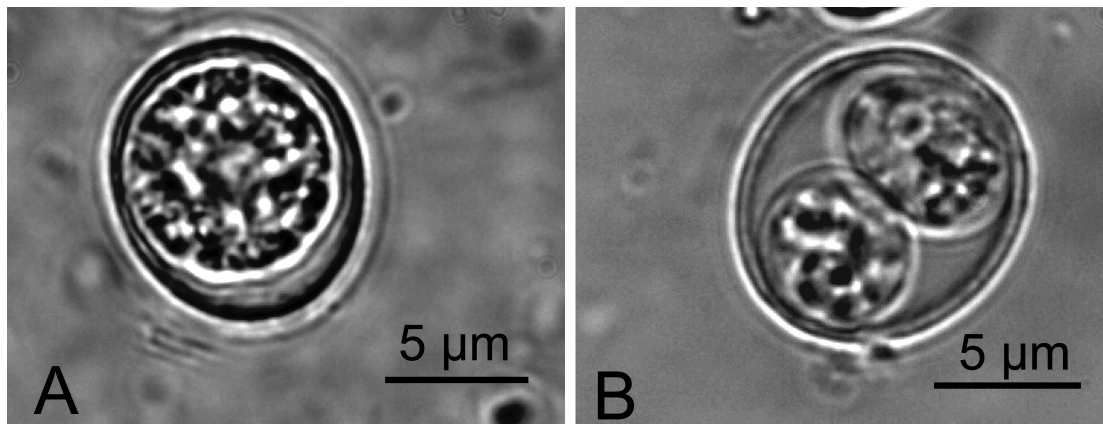


Fig 1—*Toxoplasma gondii* oocyst shed by naturally infected cat. Light micrograph of unsporulated oocysts (A) and sporulated oocysts contain 2 sporocysts (B).

RESULTS

Putative *T. gondii* oocysts isolated from naturally infected cats

Fecal samples of 5/115 (4%) cats revealed 9-14 µm-diameters *T. gondii*-like oocysts. Sporulated oocysts were ellipsoidal or ovoid in shape, 14.1-12.6 × 13.6-11.7 µm (average 13.6 × 12.4 µm, $n = 35$) in size. The smooth colorless wall was devoid of micropyle. No oocyte residuum or polar granule was present in the oocyst. The ovoid sporocysts were 8.7-6.3 × 7.6-5.3 µm (average 7.4 × 6.3 µm, $n = 24$) in size. There was no Stieda body but a granular sporocyst residuum was present (Fig 1).

PCR identification of putative *T. gondii* oocysts from naturally infected cats

Genomic DNA extracted from putative *T. gondii* oocysts isolated from each positive cat fecal sample served as template for PCR amplification. *T. gondii*-specific primer set, Tox4/Tox5, yielded an amplicon of expected size (~ 520 bp). However, CT1/HhamITS and Hham3R/Hham34F primers specific for *H. Hammondii* did not produce any amplicon. Sequence of 529 bp fragments (KX781159)

was 99% identical (using NCBI BLAST program) to GenBank *T. gondii* sequences (KC607824, DQ779189, EF195646, DQ779192, DQ779191, AF146527, DQ779196, DQ779193, DQ779190, and DQ779188).

Experimental infection

Experiment 1. One cat and 2 KM mice fed with *T. gondii* oocysts were successfully infected. Tissue cysts were present in brain. Using light microscopy, cyst sizes ranged from 30-50 µm ($n = 15$). Cyst walls were thin (< 1 µm) and surrounded several hundreds of slender bradyzoites (Fig 2A). No cysts were found in brains of control animals. None of the animals excreted oocysts in feces during the 60-day observation period, and obvious clinical symptoms were not seen in the infected animals.

Experiment 2. All cats that were fed tissues of infected cat and KM mice from Experiment 1 excreted *T. gondii* oocysts in their feces after a prepatent period of 6 and 8 days, respectively. Oocysts recovered from feces of experimentally infected cats were fully sporulated after 3 days.

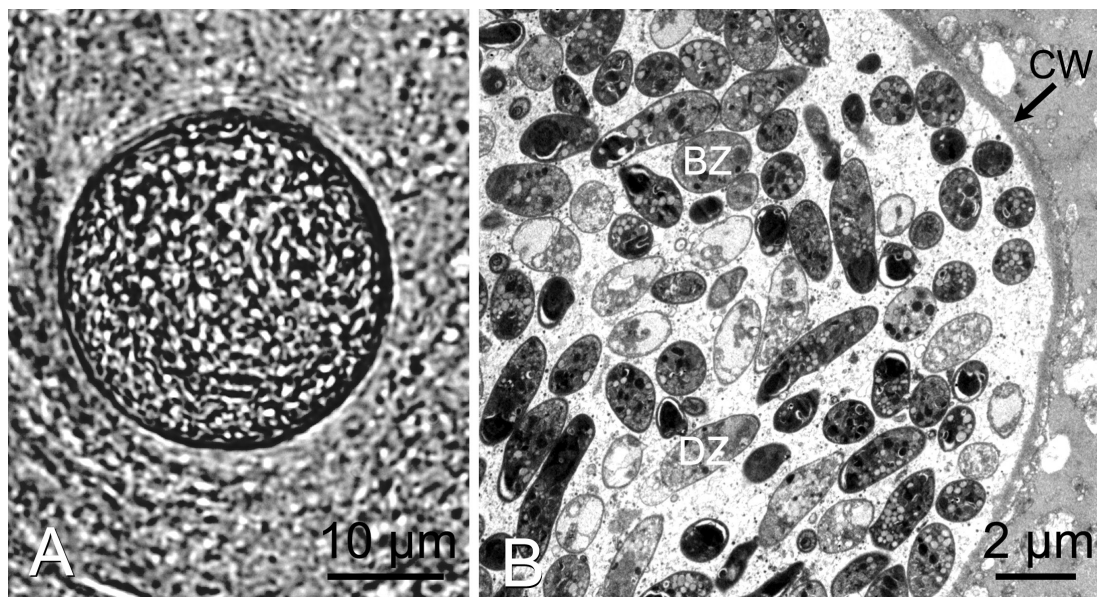


Fig 2—*Toxoplasma gondii* cyst in the brain of an infected KM mouse. (A) Light micrograph of a cyst in the brain of a KM mouse fed on *T. gondii* oocysts from naturally infected cat. (B) Electron micrograph of cyst in the brain of a KM mouse fed on carcasses of KM mice infected with *T. gondii* oocysts. BZ, bradyzoite; CW, cyst wall; DZ, disintegrating bradyzoite.

Sporulated oocysts measured 14.2-11.5 x 13.2-11.0 μm (average 13.5 x 12.3 μm, $n = 30$) in size, and morphology was indistinguishable from oocysts from feces of naturally infected cats. No oocysts were found in fecal samples of control cats. No obvious clinical symptoms were observed in infected cats.

Experiment 3. Tissue cysts were found in the brains of KM mice fed carcasses of infected KM mice from Experiment 1. Ultrastructural examination showed tissue cyst surrounded by a relatively thin cyst wall (0.4-0.6 μm). The honeycombed cyst wall enclosed hundreds of crescent-shaped bradyzoites, measuring 4.5 x 1.3 μm ($n = 12$), with a number of bradyzoites appearing to be disintegrating. Bradyzoites had a pointed anterior end and a rounded posterior end. An apical complex was situated at the anterior end and the nucleus at the posterior end (Fig 2B).

Cat fed tissues of infected mice excreted *T. gondii* oocysts in feces. However, no oocysts were found in fecal samples of the control cat. No obvious clinical symptoms were observed in infected animals.

Genotype analysis of *T. gondii* KM isolate collected from experimentally infected cat

Amplification of *GRA6* resulted in a single amplicon for *T. gondii* KM isolate in this study. Three clones of the PCR product were sequenced, and the sequences were 773 bp and shared 100% identity, so only clone 1 was deposited to GeneBank (KX781158). Alignment of the sequence with 9 reference strains sequences revealed that the *T. gondii* KM isolate was identical with that of Beverley and ME49 strains (genotype II) (Table 1). Amplification of *SAG2* resulted in a single amplicon, and 3 clones of which were sequenced. The sequences were 1,315 bp and shared 100% identity. Clone 1 was submitted to

Table 1
Nucleotide polymorphism of *Toxoplasma gondii* GRA6.

Strain	Position of variable nucleotide																																						
	41	71	106	162	170	171	195	304	336	561	575	576	614	635	637-655																								
RH	C	G	C	G	A	A	C	A	T	A	G	A	G	T	C	G	G	A	G	C	A	G	C	A	G	A	G	A	G	A	C	A	C	A	C	T	A		
M7741	C	G	
NED	.	T	T	A	.	.	.	T	C	.	C	.	C	G	
TONT	.	T	T	A	.	.	T	C	.	C	.	C	G	
RUB	.	T	.	.	.	T	T	C	C	G	C	
FOU	.	T	.	C	.	.	T	C	.	C	A	G	G	.	.		
CASL	.	T	.	A	.	.	T	C	.	C	A	G	G	.	.		
MAS	T	T	T	C	G	G	.	G	A	T
BEV	T	T	.	.	.	G	T	C	.	G	.	G	G	G	.	G	A	T
ME49	T	T	.	.	.	G	T	C	.	G	.	G	G	G	.	G	A	T
KM isolate	T	G	T	C	.	G	.	G	G	G	.	G	A	T

(.) Nucleotide identical to RH. (-) Deletion. GRA6 GenBank accession numbers are: AF239283 (RH), AF239284 (BEVERLEY), AF239285 (ME49), AF239286 (NED), AF239287 (M7741), AF239288 (FOU), AF239289 (MAS), AF239290 (RUB), AF239291 (CASTELLS), AF239292 (TONT), KX781158 (KM isolate).

GenBank (KX781160), that, when aligned with 7 reference strains sequences, was identical with the Beverley strain (Table 2). Taken together, the genotype of *T. gondii* KM isolate was characterized as genotype II.

DISCUSSION

Oocysts of *T. gondii* are structurally similar to those of *H. Hammondii* that also have cats as definitive hosts. To identify *T. gondii* oocysts in feline feces, the following criteria were used in this study: (i) detection of *T. gondii* specific 529 bp amplicon, (ii) recovery of *T. gondii* cysts from mice and cat tissues, and (iii) infectivity of cysts to mice. This survey indicated 4% shedding *T. gondii* oocysts, higher than the occurrence in North America (0.007%) (Dubey *et al*, 1977), Canada (1%) (Stojanovic and Foley, 2011), Finland (1.5%) (Jokelainen *et al*, 2012) and European countries (0.11%) (Schaes *et al*, 2008a), but lower than in Ethiopia (19%) (Dubey *et al*, 2013). The prevalence of this parasite based on monitoring anti-*T. gondii* antibodies or *T. gondii* DNA from cats ranged from 14.1% to 79.4% in different regions of China (Yu *et al*, 2006; Dubey *et al*, 2007; Tian *et al*, 2014). In general, information on the prevalence of cats shedding *T. gondii* oocysts is sparse. To the best of our knowledge, this is the first report in China of *T. gondii* identified in naturally infected cat feces.

Genetic studies have identified 3 main clonal lineages, namely, type I (mouse virulent), type II and

Table 2
Nucleotide polymorphism of *Toxoplasma gondii* SAG2.

Strain	Position of variable nucleotide																		
	29	72	272	301	411	412	423	432	440	549	550	551	674	869	998	1067	1119	1211	1231
CASL	T	G	T	G	G	C	G	C	T	G	G	T	A	A	G	C	G	C	T
RUB	.	T	.	A	A	.	.	G	.	-	-	-	.	.	C	.	A	.	.
MAS	.	T	A
C56	C	T	.	A	A	.	C	G	.	-	-	-	.	.	C
NED	C	T	.	A	A	.	C	G	.	-	-	-	.	.	C
S48	.	T	.	A	A	.	C	G	.	-	-	-	.	.	C
BEV	.	T	G	A	A	A	G	C	C	T	.	G	C
KM isolate	.	T	G	A	A	A	G	C	C	T	.	G	C

(.) Identical to CASL. (-) Deletion. SAG2 GenBank accession numbers are: AF249697 (BEVERLEY), AF249698 (C56), AF357579 (NED), AF357580 (MAS), AF357581 (RUB), AF357582 (CASTELLS), AF357577 (S48), KX781160 (KM isolate).

type III (both mouse non-virulent) (Sibley *et al*, 1992; Howe and Sibley, 1995). In the present study, *GRA6* and *SAG2* nucleotide sequences from *T. gondii* KM isolate were identical with type II genotype (BEVERLEY and ME49 strains). In addition, *T. gondii* cysts were found in the brains of mice, although no obvious clinical symptoms were evident, consistent with the findings that type II strains are usually avirulent in mouse (Sibley and Boothroyd, 1992; Li *et al*, 2014). The predominance of *T. gondii* genotype II in cats has been reported in European countries (100%) (Schaes *et al*, 2008a), Turkey (86.3%) (Can *et al*, 2014), and Spain (74%) (Montoya *et al*, 2008).

Based on multi-locus polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, at least 6 genotypes of *T. gondii* isolates have been identified from feline tissues in different geographic regions of China, namely, Toxoids #1 (type II), ToxoDB #2 (type III), ToxoDB #3, ToxoDB #9, ToxoDB #17, ToxoDB #20, with ToxoDB #9 regarded as the major genotype (Dubey *et al*,

2007; Chen *et al*, 2011; Qian *et al*, 2012; Li *et al*, 2014; Tian *et al*, 2014; Yang *et al*, 2015). Among these genotypes, ToxoDB #1, ToxoDB #3, ToxoDB #9, and ToxoDB #20, have the same type II restriction enzyme patterns at *GRA6* and *SAG2* loci. Unfortunately, owing to the lack of the complete sequences of *SAG2* and *GRA6* from these isolates found in China, we could not compare *T. gondii* KM isolate with those of the previous studies.

It has been suggested that *T. gondii* type II isolates are more likely to result in clinical toxoplasmosis (Howe and Sibley, 1995). For example, in France, of 86 *T. gondii* isolates obtained from patients with toxoplasmosis, 85% were type II, 8% Type I, 2% type III, and 5% atypical (Ajzenberg *et al*, 2002). It is worth noting that the first fatal case of toxoplasmosis in China (and Asia) was reported from an immunocompetent adult in Kunming City during the period of this study (Yang *et al*, 2013), but the genotype of the pathogen and pathway of infection were not indicated. The proximity of this city to the

Southeast Asian region indicates the necessity to be more deeply concerned with the prevalence and population genetics of *T. gondii* in domestic and wild animals in this part of Asia.

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