Isolation and characterization of viable *Toxoplasma gondii* isolates revealed possible high frequency of mixed infection in feral cats (*Felis domesticus*) from St Kitts, West Indies

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SUMMARY

Cats are essential in the epidemiology of *Toxoplasma gondii* because they are the only hosts that can excrete the environmentally resistant oocysts in nature. Samples of serum, feces, and tissues from feral cats from St Kitts, West Indies were examined for *T. gondii* infection. Antibodies to *T. gondii* were assayed by the modified agglutination test, and found in 71 of 96 (73·9%) of cats with titres of 1 : 10 in six, 1 : 20 in six,1 : 40 in seven,1 : 80 in three, 1 : 160 in 10, 1 : 320 in 13, 1 : 640 in nine, and 1 : 1,280 or higher in 17. Tissues of 10 cats were bio-assayed in mice. *Toxoplasma gondii* was isolated from tissues of 7 cats; from hearts of 6, from tongue of 5, and brains of 3 cats. All 7 isolates were avirulent for mice. *Toxoplasma gondii* oocysts were not found in the feces of 51 cats. Genotyping of these 7 *T. gondii* isolates by 10 multi-locus PCR-RFLP markers, including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and an apicoplast marker, Apico, revealed 4 genotypes, including clonal Type II, Type III and 2 unique genotypes. Five of the 7 cats had infection with 2 genotypes, indicating high frequency of mixed infection in the cat population on the St Kitts island.

Key words: *Toxoplasma gondii*, *Felis domesticus*, Feral, St Kitts, West Indies, genotype.

INTRODUCTION

Toxoplasma gondii infections are widely prevalent in human beings and other animals worldwide (Dubey and Beattie, 1988). Humans become infected postnatally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts from the environment. Until recently, *T. gondii* was considered to have low genetic diversity. We have recently found that the isolates of *T. gondii* from Brazil are biologically and genetically different from those in North America and Europe (Dubey *et al.* 2002; Lehmann *et al.* 2006; Dubey *et al.* 2007*a*). *Toxoplasma gondii* isolates from asymptomatic chickens from Brazil were more pathogenic to mice than isolates from Europe or North America, irrespective of the genotype (Dubey *et al.* 2006*a*). Therefore, strains isolated from chickens in Brazil possess greater genetic diversity than those identified in Europe and North America.

Cats are essential in the life cycle of *T. gondii* because they are the only hosts that can excrete the environmentally-resistant oocysts in nature (Dubey and Beattie, 1988). We have started to study genetic diversity of *T. gondii* isolates from cats but it is difficult to obtain samples from feral domestic cats (*Felis domesticus*). Until now, data were obtained from domestic cats from Brazil (Dubey *et al.* 2004; Pena *et al.* 2006), The Peoples Republic of China (Dubey *et al.* 2007*c*), and Colombia (Dubey *et al.* 2006*b*). In the present study, the seroprevalence and the isolation and genetic characterization of *T. gondii* from feral cats on St Kitts, West Indies are reported.

MATERIALS AND METHODS

Naturally-infected cats

The St Kitts island is located in the Eastern Caribbean at 17 20 North, 62 45 West with habitats ranging from dry areas to rainforests. The island is 179 km^2 with a population of approximately 35 000.

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Table 1. Isolation of *Toxoplasma gondii* from feral cats from St Kitts

Cat no.	MAT titre	Isolation in mice[a]			*Toxoplasma gondii* isolate designation
		Heart	Brain	Tongue	
40	320	3	0	0	TgCatStK1 – unique type
44	320	4	0	4	TgCatStK2 – Type III, – Type II?
45	1280	4	0	4	TgCatStK3 – Type II – Type III
46	640	2	0	4	TgCatStK4 – Type III, – Type II?
72	320	ND[b]	2	ND	TgCatStK5 – Type II? III?
85	320	4	4	4	TgCatStK6 – Type III – Type II?
98	1280	1[c]	1	4	TgCatStK7 – unique type

[a] No. of mice infected with *T. gondii* from 4 mice inoculated with feline tissues.
[b] Mice were killed accidentally 34 day p.i. and not examined for *T. gondii* infection.
[c] One mouse died on 16 day p.i.

In total, 96 feral cats (46 males, 50 females; 14 <6 months of age, and 82 adults) within 10 km radius of Basseterre, St Kitts were captured from their original colonies in cages and identified by ear notching during 2005 and 2006. These cat colonies represent the most populated region of the island in and near the capital, Basseterre. During the day 2/3 of the island population commute to work in Basseterre and after several hours return to their homes in the rural areas on the island. All procedures involving handling, sample collection, surgery and euthanasia were performed according to the Institutional Animal Care and Use Committee (IACUC) from Ross University, School of Veterinary Medicine. Cats were housed and fed *ad libidum* at the Ponds Veterinary Hospital, a local veterinary clinic owned by Dr Burnell Nisbet. For easier handling and safety precautions all feral cats were anesthetized using 50% ketamine, 50% zylazine solution (1 ml/cat) administered intramuscularly. Approximately 3 ml of blood were obtained from the jugular vein, labelled, centrifuged, and serum stored at −20 °C. Feces from the rectum of 51 cats were obtained for parasitological examination. All cats were tested for Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) infections using the SNAP Combo Test kits (Idexx Laboratories, Westbrook, Maine, USA). None of the 96 cats were positive for FeLV. Fifteen of the 96 cats were positive for FIV and these 15 cats were euthanized using an overdose (>50 mg/kg) of pentobarbital. All cats were clinically examined and their general health status determined. Most of the FIV-positive animals were adult males showing scratches and bites, indicating a more aggressive behaviour, but in a good health condition otherwise. The remaining 81 cats were dewormed, vaccinated, spayed/neutered and returned to their original colonies approximately 5 days after surgery.

Samples of sera from 96 cats, feces from 51 cats, and tissues from 10 cats were shipped with cold packs from St Kitts to Beltsville, Maryland for *T. gondii* evaluation.

Serological examination for T. gondii

Two-fold serial dilutions were made (1:10 to 1:1280) and tested with a modified agglutination test (MAT), as described previously (Dubey and Desmonts, 1987).

Bioassay of feline tissues for T. gondii *infection*

Brain, heart, and tongue of each of the 10 cats were bio-assayed for *T. gondii* infection in out-bred female Swiss Webster mice obtained from Taconic Farms, Germantown, New York, as described by Dubey *et al.* (2002). Each tissue (20–50 g) was homogenized individually, digested in an acidic pepsin solution, neutralized, centrifuged (Dubey, 1998); the homogenate was inoculated subcutaneously (s.c.) into 5 mice (Table 1). The recipient mice were examined for *T. gondii* infection. Tissue imprints of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 40–42 post-inoculation (p.i.) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the MAT. Mice were killed 6 weeks p.i. and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988). The inoculated mice were considered infected with *T. gondii* when tachyzoites or tissue cysts were found in tissues.

Bioassay of feline feces for T. gondii *oocysts*

Feces (1–5 g) from the rectum of 51 cats were floated in sugar solution (sp. gr. 1·18), and the float was examined microscopically for oocysts. Approximately

0·5 ml of the float from the very top of the tube was mixed with 10 ml of 2% aqueous sulfuric acid, aerated at room temperature for 1 week and sent to Beltsville for bioassay. The acid in the sample was neutralized, centrifuged, and inoculated orally into mice (Dubey *et al*. 2005).The recipient mice were examined for *T. gondii* infection.

Genetic characterization

Toxoplasma gondii DNA was extracted from the tissues of all 46 infected mice (Table 1) and strain typing was performed using 10 PCR-RFLP genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico (Dubey *et al*. 2006*a*, 2007*c*; Su *et al*. 2006). To confirm mixed infections, PCR products were first sequenced from both ends using sequencing primers (forward and reverse). If the DNA sequence chromatogram showed 2 nucleotide peaks at the expected single nucleotide polymorphism (SNP) site, then the PCR products were cloned by TA cloning using pGEM – T Easy vector system (Promega, Madison, Wisconsin) following the manufacturer's instructions. Ten to 25 clones for each isolate are picked to expand in 1 ml of LB broth with 100 μg/ml ampicillin. These clones were genotyped again and representative clones were sequenced to confirm the existence of 2 different DNA sequences. Three loci including SAG2, c22-8 and L358 were sequenced for the representative isolates from each cat that had potential mixed infection based on PCR-RFLP results. Here, the SAG2 is the marker based on the 5′-end of the gene sequence (Su *et al*. 2006). Reference strains RH88, PTG and CTG for Type I, II and III lineages, respectively, were also included for sequencing. The primers used for sequencing are: SAG2-SqF, TAGCTTTC-AAGACCGCACCT and SAG2-SqR, CTGCT-TGCGATTCTGTGTGT for SAG2; c22-8SqF, AAGGATCGGGGAAAGTGTCT and c22-8SqR, GCGAACCTTCTGTCATCTCC for c22-8; and L358-SqF, ATGTCCTCTTTCTGCCTTCG and L358-SqR, GGAGAAAGCGAAACCTTCCT for L358.

RESULTS

Antibodies to *T. gondii* were found in the sera of 71 of 96 (73·9%) of cats with titres of 1 : 10 in six, 1 : 20 in six, 1 : 40 in seven, 1 : 80 in three, 1 : 160 in 10, 1 : 320 in 13, 1 : 640 in nine, and 1 : 1, 280 or higher in 17.

Toxoplasma gondii was isolated from tissues of 7 of the 10 cats; from the hearts of 6, tongues of 5, and the brains of 3 cats (Table 1). All 7 isolates of *T. gondii* were avirulent for mice (Table 1). *Toxoplasma gondii* was not isolated from the feces of cats from St Kitts.

Genotyping of the 7 isolates revealed 4 genotypes, including clonal Type II, III and 2 unique genotypes. Tissues from 5 of the 7 cats, when inoculated into mice, gave rise to infections with more than 1 strain of *T. gondii* (Table 2). Isolates TgCatStK1 and TgCatStK7 each had a unique genotype that is not found from South America isolates studied to date. TgCatStK2, TgCatStK4 and TgCatStK6 each had a Type III genotype with the possibility of mixed infection with Type II genotype. TgCatStK3 clearly showed a mixed infection of Type II and III genotypes. TgCatStK5 showed the possibility of a mixed infection with Type II and III genotypes.

To confirm mixed infection, 1 representative isolate from each cat with a potential mixed infection was sequenced at 3 loci, including SAG2, c22-8 and L358. DNA sequencing data from at least 2 of the 3 loci confirmed the PCR-RFLP data. An example of the DNA chromatogram trace data for TgCatSk2 at locus SAG2 is summarized in Fig. 1. It is shown that 5 of the 7 cat isolates, including TgCatStk2, 3, 4, 5 and 6 have mixed infection of 2 genotypes. The mixed infection was further confirmed by TA cloning of PCR products (marker SAG2 or c22-8), genotyping 10–25 individual clones for each isolate and then sequencing representative clones. Four of the above 5 isolates, including TgCatStk 2, 3, 4 and 5, were confirmed to have 2 different sequences. TgCatStk6 was not done due to an insufficient amount of DNA samples. Figure 2 shows the results of genotyping individual clones from TA cloning of TgCatStk4 at the SAG2 locus.

DISCUSSION

The results indicate that the environment on St Kitts is highly contaminated with *T. gondii* oocysts already shed by the sero-positive cats. Cats shed *T. gondii* oocysts only for a short time (<1 week) and at any given time less than 1% were shedding oocysts (Dubey, 2004). Cats usually develop antibodies to *T. gondii* 1–2 weeks after they have shed oocysts (Dubey, 2004). This may be the reason why oocysts were not detected in feces of any of the 51 cats examined in the present study. Infected cats can release millions of *T. gondii* oocysts into the environment and people become exposed by ingesting the oocysts or by eating undercooked meat from animals which have ingested oocysts and developed tissue cysts. Although there are no published data for St Kitts, our study suggests that toxoplasmosis might be an important disease on this island and health workers on this and neighbouring islands should be alerted to this possibility.

In the present study *T. gondii* was isolated from 7 of the 10 cats that were also infected with the FIV. These 7 cats appeared to have no clinical signs and there is no evidence that the 70% isolation rate of *T. gondii* from sero-positive cats with dual infection with *T. gondii* and FIV is different than cats in the general population (Dubey *et al*. 2004; Pena *et al*.

Table 2. Genotyping of *Toxoplasma gondii* isolates from feral cats from St Kitts, West Indies

ID and Genotype	Cat no.[a]	SAG1	5′+3′ SAG2[b]	SAG2[d]	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
TgCatStK1 – unique type	40H	II or III(3)[c]	III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	I(3)	III(3)
TgCatStK2	44 H	II or III	II	II	II	II	II	II&III	II&III	II	II	II&III
– Type III		II or III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)
– Type II?		II or III	II&III	II&III	III	III	III	III	III	III	ND[e]	III
	44 T	II or III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)
		II or III(2)	II&III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)
TgCatStK3	45-H	II or III	II&III	III	II&III	III	III	III	II	II&III	III	II&III
– Type II		II or III	II&III	III	II&III	II	III	II	II	II&III	III	II&III
– Type III		II or III	II&III	II&III	II	II	II	II	II	II&III	ND	II&III
		II or III	II	II	II	II	II	II	II	II	II	II
	45-T	II or III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	ND(3)	III(3)	III(3)	III(3)
		II or III	II&III	III	III	III	III	III	III	III	III	III
TgCatStK4	46-H	II or III	III	III	III	III	III	III	III	III	III	III
– Type III,	46-T	II or III(3)	II&III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	III(3)	III(3)
– Type II		II or III	II&III	II&III	II&III	III	III	III	III	III	III	III
TgCatStK5	72-B	II or III	III	II&III	II&III	III	III	II&III	ND	II&III	ND	ND
– Type II		II or III	III	II&III	II&III	III	III	II	ND	II&III	II	ND
– Type III												
TgCatStK6	085-H[f]	II or III(2)	II&III(2)	III(2)	II&III (2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)	III(2)
– Type III		ND	III	III	III	III	III	III	ND	III	III	ND
– Type II	085-T	II or III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)
	085-B	II or III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)	III(4)
TgCatStK7	98-T	I(4)	I(4)	I(4)	I(4)	I(4)	III(4)	II(4)	III(4)	III(4)	I(4)	III(4)
– unique type	98-B	I	I	I	I	I	III	II	III	III	I	III
	98-H	I	I	I	I	I	III	II	III	III	I	III

[a] Isolates derived from B = Brain, H = Heart, T = Tongue.
[b] *SAG2* marker based on 5′- and 3′-ends of the gene sequence (Howe *et al*. 1997).
[c] No. of mice with the same genotype.
[d] A new *SAG2* marker based on the 5′-end of the gene sequence (Su *et al*. 2006).
[e] ND, no data.
[f] Not enough DNA for genotyping from 1 of the 4 infected mice.

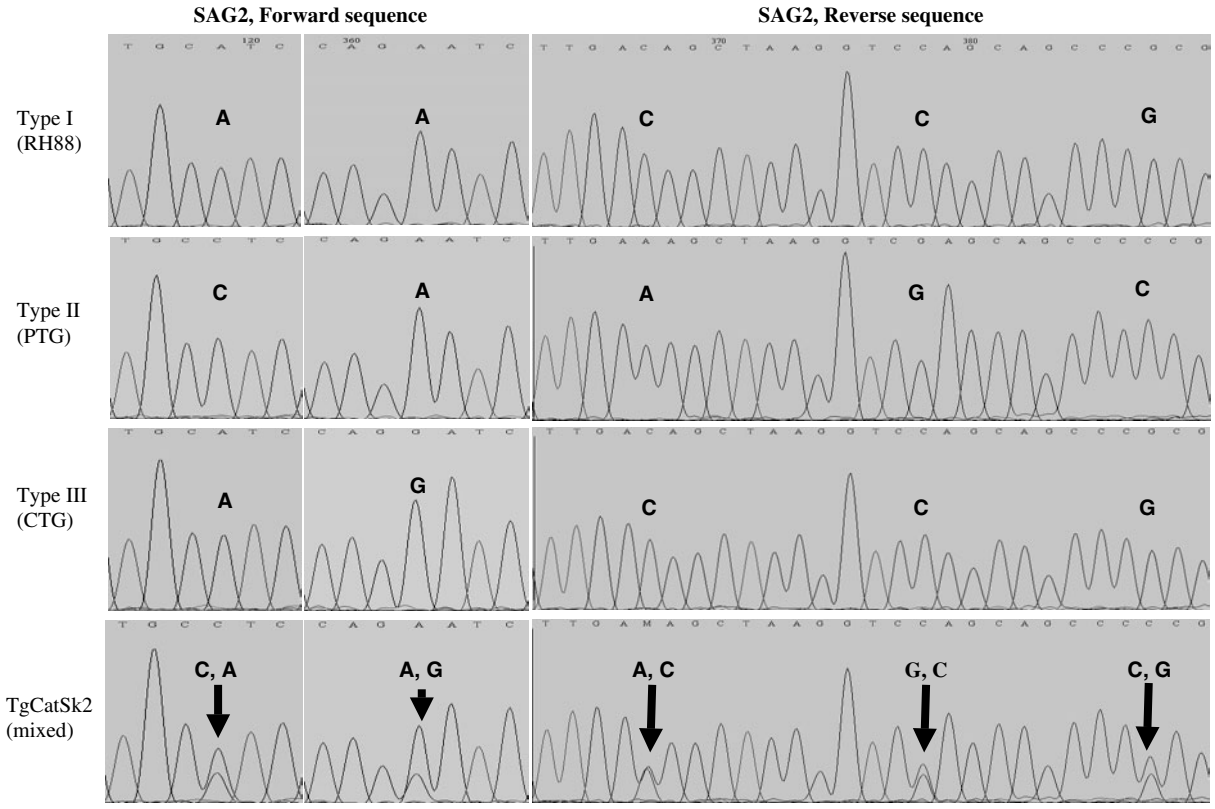

Fig. 1. Summary of DNA trace data that contain the mixed nucleotides at SAG2 locus. The data were based on the forward and reverse sequences of SAG2 locus. The polymorphic sites were indicated by the letters of the nucleotide. Reference strains RH88, PTG and CTG represent the clonal Type I, II and III lineages, respectively. TgCatSk2 with potential mixed infection showed 2 nucleotides at 5 positions that matched exactly to the polymorphic sites among the 3 reference strains.

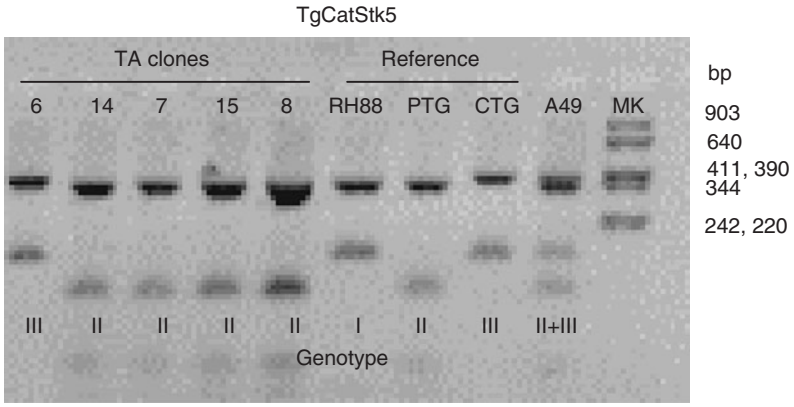

Fig. 2. Genotyping of individual clones from TA cloning of TgCatStk5 at the SAG2 locus. Sample A49 is the direct PCR product showing mixed genotypes (types II and III). Samples 6, 7, 8, 14 and 15 are clones derived from A49 by TA cloning. Sample 6 shows a type III allele, whereas samples 7, 8, 14, and 15 have type II alleles. DNA sequencing of samples 6 and 14 confirmed the mixed infection of types II and III alleles. MK is the DNA marker. RH88, PTG and CTG are reference type I, II and III lineages, respectively.

2006). Viable *T. gondii* was isolated from 68·5% of 54 sero-positive (Dubey *et al*. 2004) and 66·2% of 71 sero-positive (Pena *et al*. 2006) cats from Brazil. However, cats from Brazil were not tested for the FIV infection. The FIV is morphologically and biologically similar to the human immunodeficiency virus (Levy *et al*. 1998); there are no indications that the FIV infection modifies the course of latent *T. gondii* infection in cats (see Dubey and Carpenter, 1993).

Until recently, *T. gondii* was considered to have an affinity for encystment in neural tissue but this assumption is based on infections in mice. Results of the present study indicate that the muscular tissues

in cats are more heavily parasitized with *T. gondii* than the brain, which supports earlier findings (Dubey *et al*. 2004, 2006*b*).

Genotyping of the 7 cat isolates from St Kitts identified 4 genotypes. Two genotypes are unique and were not identified from isolates collected from a variety of hosts in South America including Brazil, Colombia, Chile, Costa Rica, Nicaragua and Guyana (see Dubey *et al*. 2007*a*, *b*). The other 2 genotypes are identical to the clonal Type II and III lineages that are predominant in North America and Europe. This study showed that there is potentially a high percentage of mixed infections among the feral cats. To identify mixed infections with both Type II and Type III genotypes with ultimate certainty, the actual parasites from the infected tissues need to be isolated, cloned and re-examined at all PCR-RFLP loci. Unfortunately, these infected tissues are no longer available at this point and further experiments are not possible.

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