Isolation and RFLP Genotyping of Toxoplasma gondii in Free-Range Chickens (Gallus domesticus) in Grenada, West Indies, Revealed Widespread and Dominance of Clonal Type III Parasites

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ISOLATION AND RFLP GENOTYPING OF *TOXOPLASMA GONDII* IN FREE-RANGE CHICKENS (*GALLUS DOMESTICUS*) IN GRENADA, WEST INDIES, REVEALED WIDESPREAD AND DOMINANCE OF CLONAL TYPE III PARASITES

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ABSTRACT: The objectives of the present cross-sectional study were to isolate and genotype *Toxoplasma gondii* in free-range chickens from Grenada, West Indies. Using the modified agglutination test, antibodies to *T. gondii* were found in 39 (26.9%) of 145 free-range chickens with titers of 25 in 7 chickens, 50 in 6 chickens, 100 in 2 chickens, and 200 or higher in 24 chickens. The hearts of the 39 seropositive chickens were bioassayed in mice; viable *T. gondii* was isolated from 20 and further propagated in cell culture. Genotyping of *T. gondii* DNA extracted from cell-cultured tachyzoites using the 10 PCR-restriction fragment length polymorphism (RFLP) markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico revealed 4 genotypes, including ToxoDB PCR-RFLP no. 2 (Type III), no. 7, no. 13, and no. 259 (new). These results indicated that *T. gondii* population genetics in free-range chickens seems to be moderately diverse with ToxoDB no. 2 (Type III) as the most frequent (15/20 = 75%) compared to other genotypes in Grenada.

Infection due to the zoonotic protozoan parasite *Toxoplasma gondii* is worldwide in distribution. All warm-blooded animals including mammals and birds are suceptible to *T. gondii* (Robert-Gangneux and Dardé, 2012). Cats are important in the life cycle of *T. gondii* because they are the only known definitive hosts capable of shedding environmentally resistant oocysts in nature (Dubey, 2010). Sporulated oocyts serve as a source of infection for a wide range of intermediate hosts.

Free-range chickens are important in the epidemiology of *T. gondii* because they feed from the ground, thus getting exposed to different genotypes of *T. gondii* oocyts. Serological surveys revealed high *T. gondii* prevalence backyard chickens worldwide (Dubey, 2010).

Initially, *T. gondii* was considered clonal with low genetic diversity and grouped into 3 major lineages: types I, II, and III (Howe and Sibley, 1995). However, research has revealed that the genetic diversity of *T. gondii* is far greater than previously appreciated; the population structure of *T. gondii* is strongly subdivided by geographic region and by the existence of non-clonal lineages in some regions such as South America (Shwab et al., 2014). Importantly, some of these South American lineages are associated with severe ocular disease, suggesting that differences in clinical severity may be influenced by the parasite genotype (Khan et al., 2006).

There is limited information about genotypes and genetic diversity of *T. gondii* in free-range chickens in the Caribbean region. The objectives of this study were to isolate and speciate *T. gondii* in free-range chickens from Grenada by using the 10 PCR-RFLP markers: SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Su et al., 2010).

MATERIALS AND METHODS

Location of the study

Grenada is a Caribbean island located 12 degrees north of the equator. This island consists of a warm, humid climate that averages annual temperatures ranging from 24 to 31 C. The 2 bodies of water that surround Grenada are the Caribbean Sea to the west and the Atlantic Ocean to the east.

Naturally infected backyard chickens

One hundred and forty-five backyard chickens from 5 parishes of Grenada were purchased, captured, and transported to the veterinary pathology diagnostic laboratory for necropsy, following humane euthanasia. Heart and blood were collected from each chicken. The blood was centrifuged at 2,500 *g* to separate serum. Sera and hearts were stored at 2 C for 2–3 days before they were shipped to the Animal Parasitic Diseases Laboratory (APDL), United States Department of Agriculture, Maryland, in order to evaluate *T. gondii* infection.

Serological testing

Sera from free-range chickens were tested for IgG antibodies to *T. gondii* by the modified agglutination test (MAT) as described by Dubey (2010). Sera were diluted 2-fold serially from 1:25 to 1:200. Titers equal or greater than 1:25 are considered positive. The MAT was found to be highly accurate for the detection of *T. gondii* infection in chickens (Dubey et al., 2016a).

Bioassay in mice

Heart samples from the 39 seropositive free-range chickens were bioassayed in outbred albino Swiss mice (National Cancer Institute, Bethesda, Maryland) following a previously published method (Dubey, 2010). For this, whole myocardial tissue (~10–15 g) from each backyard chicken was homogenized in normal

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TABLE I. *Toxoplasma gondii* seroprevalence and mouse bioassay of chicken samples from Grenada.

Assay	Origin	Total sampled/ bioassay	MAT titer					Total positive	%
			<25	25	50	100	≥200		
Serology	St. Andrew	51	43	0	4	0	4	8	15.7
	St. Georges	50	41	2	1	1	5	9	18
	St. John	22	10	4	0	1	7	12	54.5
	St. Mark	12	4	1	1	0	6	8	66.7
	St. Patrick	10	8	0	0	0	2	2	20
	Total	145	106	7	6	2	24	39	26.9
Mouse bioassay	St. Andrew	7	0	0	4 (4)*	0	3 (3)	7	100
	St. Georges	9	0	2	1	1 (1)	5 (3)	4	44.4
	St. John	8	0	0	0	1 (1)	7 (4)	5	62.5
	St. Mark	6	0	0	0	0	6 (2)	2	30
	St. Patrick	2	0	0	0	0	2 (2)	2	100
	Total	32	0	2 (0)	5 (4)	2 (2)	23 (14)	20	62.5

* Total bioassay (total isolated).

saline, digested in pepsin, centrifuged at 1,200 *g*, suspended in saline (0.85% aqueous NaCl), and neutralized with sodium bicarbonate and centrifuged at 1,200 *g*. The sediment was resuspended in normal saline containing 1,000 units of penicillin G and 100 μg of streptomycin per ml and the homogenate inoculated into 2 outbreed Swiss Webster mice. The mice that survived were bled 2 mo later and their sera examined for IgG antibodies to *T. gondii* using MAT at a serum dilution of 1:25. Brains of all surviving mice were examined for the presence of tissue cysts by brain squash smear preparations. Mice with no demonstrable antibodies to *T. gondii* and tissue cysts were considered not infected.

In vitro cultivation of *T. gondii* and RFLP genotyping

Mouse brain tissues infected with *T. gondii* isolates were seeded on to African green monkey kidney fibroblast cells (CV-1 cell line) culture flasks, and tachyzoites were harvested from the medium. DNA was extracted from cell-cultured tachyzoites, and genotyping was performed using 10 PCR-RFLP genetic markers, SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico, as described previously (Su et al., 2010). Appropriate positive and negative controls were included in each electrophoresis gel run. Designation of the *T. gondii* genotypes was done by referring to a *T. gondii* data base (http://toxodb.org/), as described by Kissinger et al. (2003).

Ethical approval

Ethical approval to conduct this study was granted by the institution of animal care and use committee (IACUC) at St. George's University (IACUC number 13016-R) and by the institutional of animal care and use protocol committee of the United States Department of Agriculture.

RESULTS

Out of the 145 free-range chickens, 39 (26.9%) were seropositive for *T. gondii* (Table I). Bioassay in mice of the 32 seropositive chicken hearts yielded 20 isolates designated as TgCkGr37 to TgCkGr57 (Table II). These isolates were further propagated in cell culture (CV-1 cells fibroblasts). Genotyping of *T. gondii* revealed 4 genotypes including ToxoDB PCR-RFLP no. 2 (clonal Type III, 15 isolates), no. 7 (atypical, 1 isolate), no. 13 (atypical, 3 isolates), and no. 259 (atypical and new, 1 isolate, Table III). Type III was significantly more prevalent than the rest of the genotypes. Phenotypically, all the isolates were avirulent for SW mice.

DISCUSSION

Free-range chickens are important in the epidemiology of *T. gondii* because they feed from the ground and their infection with *T. gondii* is considered as an indicator of the level of environmental contamination (Dubey et al., 2005).

In the previous study of *T. gondii* in free-range chickens, molecular characterization of the 35 isolates using restriction fragment length polymorphism (RFLP) at 1 locus, SAG 2, revealed 29 isolates as type III, 1 isolate as type I, and 4 isolates as type II (Dubey et al., 2005). Subsequently, only 9 out of the 35 cryopreserved isolates from the aforementioned study were revived in cell culture and evaluated further using RFLP on 11 loci (SAG 1, SAG 2, alt.SAG 2, SAG 3, BTUB, GRA 6, L 358, PK 1, C22-8, C 29-2, and Apico); revealing 4 non-clonal (atypical) and 5 type III genotypes (Rajendran et al., 2012). The present study based on a large sample size sheds more light on the genotypes and molecular diversity of *T. gondii* in free-range chickens in Grenada.

In the present study, type III appears to be the most predominant strain, but diversity of *T. gondii* can be considered to be moderate. A related study on *T. gondii* genotyping in stray dogs in Grenada reported type III as the most common and with high diversity (Dubey et al., 2013). In rats from Grenada, the only isolate was also a type III (Dubey et al., 2006). A study on molecular characterization of *T. gondii* in mongoose (*Herpestes auropunctatus*) in Grenada revealed 3 genotypes, of which one was a type III (ToxoDB no. 2), 2 ToxoDB no. 7, and 1 ToxoDB no. 216 (Choudhary et al., 2013). In addition, the study in stray dogs in Grenada revealed 1 new genotype designated ToxoDB no. 224 (Dubey et al., 2013). In the present study, we also report a new genotype (ToxoDB no. 259) in chickens. Taken together, finding of new genotypes in the current study and the previous one in stray dogs has shed more light on the population diversity of *T.*

TABLE II. *Toxoplasma gondii* isolation from myocardium of chickens from Grenada.

Chicken no.	Origin	MAT titer	Bioassay (SW)*	Isolate designation	PCR-RFLP genotype no.
5	St. John	200	2/2	TgCkGr37	7
6	St. John	200	1/2	TgCkGr38	259 (new)
12	St. John	200	2/2	TgCkGr39	2
17	St. John	100	2/2	TgCkGr40	2
18	St. John	200	2/2	TgCkGr41	2
50	St. Mark	200	1/2	TgCkGr42	2
55	St. Mark	200	2/2	TgCkGr43	2
62	St. Patrick	200	2/2	TgCkGr45	13
66	St. Patrick	200	1/2	TgCkGr46	13
210	St. Georges	200	2/2	TgCkGr47	2
219	St. Georges	100	2/2	TgCkGr48	2
272	St. Georges	200	1/2	TgCkGr49	2
338	St. Georges	200	2/2	TgCkGr50	2
319	St. Andrew	50	2/2	TgCkGr51	2
320	St. Andrew	200	2/2	TgCkGr52	2
321	St. Andrew	50	2/2	TgCkGr53	2
322	St. Andrew	200	2/2	TgCkGr54	2
323	St. Andrew	200	2/2	TgCkGr55	2
324	St. Andrew	50	2/2	TgCkGr56	13
325	St. Andrew	50	2/2	TgCkGr57	2

* No. of mice *T. gondii* positive/no. of mice inoculated.

gondii in Grenadian animals in particular and worldwide in general.

A study of toxoplasmosis in cats in St. Kitts and Nevis, West Indies, revealed types III and II, and 2 unique genotypes from the 7 isolates (Dubey et al., 2009). Similarly, a recent study based on limited molecular characterization of *T. gondii* DNA directly from tissues of small ruminants and pigs in St. Kitts and Nevis, West Indies, revealed Type III as the most predominant genotype (Hamilton et al., 2015). A recent study in dogs on the same island revealed 6 isolates of which 4 were type III and 2 were atypical (Dubey et al., 2016b).

Overall, the findings in our present study agree with the general trend that genetic diversity of *T. gondii* in Central and South America is high (Schwab et al., 2014), and interestingly the clonal type III is widespread and dominant in this area.

Regarding the virulence of *T. gondii*, type I genotype is highly virulent, whereas types II and III are not virulent in mice (Robert-Gangneux and Dardé, 2012). Recent studies investigating the genetic nature of virulent strains of *T. gondii* have shown that ROP18 and ROP5 gene allele types in *T. gondii* are highly predictive of virulence in mice (Dubey et al., 2014; Shwab et al., 2016). In the present study, none of the mice inoculated with the *T. gondii* chicken isolates from Grenada died, suggesting that all the isolates including the non-clonal ones were not virulent in mice. This is in contrast to studies in South America where the atypical strains exhibited virulence in mice and in humans (Dubey et al., 2005; Khan et al., 2006). A further molecular study on

TABLE III. PCR-RFLP genotyping of viable *T. gondii* isolates from chicken from Grenada.

Strain designation	ToxoDB PCR-RFLP genotype no.	Genetic markers										
		SAG1	(5′ + 3′) SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico
GT-1	10 (Type I)	I	I	I	I	I	I	I	I	I	I	I
PTG	1 (Type II)	II	II	II	II	II	II	II	II	II	II	II
CTG	2 (Type III)	II/III	III	III	III	III	III	III	III	III	III	III
MAS	17	u-1	I	II	III	III	III	u-1	I	I	III	I
TgCgCa1	66	I	II	II	III	II	II	II	u-1	I	u-2	I
TgCtBr5	19	I	III	III	III	III	III	I	I	I	u-1	I
TgCtBr64	111	I	I	u-1	III	III	III	u-1	I	III	III	I
TgRsCr1	52	u-1	I	II	III	I	III	u-2	I	I	III	I
Present study												
TgCkGr37	7	I	III	III	III	III	III	III	III	III	III	I
TgCkGr38	259 (new)	II or III	III	III	III	I	III	II	III	III	III	III
TgCkGr39–43, 47–55, 57	2	II or III	III	III	III	III	III	III	III	III	III	III
TgCkGr45,46, 56	13	I	I	I	I	I	III	II	III	III	I	III

virulence of *T. gondii* strains found in Grenada, targeting 4 gene loci (ROP18, ROP5, ROP16, and ROP17), is recommended.

Genotyping analysis of 88 *T. gondii* isolates in immunocompromised patients in France revealed all 3 clonal genotypes and non-clonal ones as causal agents of illness (Ajzenberg et al., 2009). Given the emerging problem of HIV infection in Grenada in particular and the Caribbean in general, *T. gondii* will continue to pose a major threat as an opportunistic infectious agent in this group of people. In addition, free-range chickens contribute significantly to household food security as a source of meat and eggs in Grenada. In light of the findings in the present study, people should be advised to adequately cook free-range chicken meat before its consumption.

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