DOI: 10.1002/vms3.870

ORIGINAL ARTICLE

WILEY

Toxoplasma gondii clonal type III is the dominant genotype identified in Grenadian pigs

Alfred Chikweto[1] | Andy Alhassan[1] | Chunlei Su[3] | Calum Macpherson[2] | Muhammad Iqbal Bhaiyat[1] | Jitender P. Dubey[4]

[1]Department of Pathobiology, School of Veterinary Medicine, St. George's University, St. George's, Grenada

[2]Windward Islands Research Foundation, St. George's University, St. George's, Grenada

[3]Department of Microbiology, University of Tennessee, Knoxville, Tennessee, USA

[4]Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center, United States Department of Agriculture, Agricultural Research Service, Beltsville, Maryland, USA

Correspondence
Alfred Chikweto, Department of St. George's, Grenada Pathobiology, School of Veterinary Medicine, St. George's University, Grenada, West Indies.
Email: achikweto@sgu.edu

Funding information
St. George's University- Grenada

Abstract

Background: *Toxoplasma gondii* is a widespread zoonotic protozoan parasite capable of infecting all warm-blooded animals. Although the genotypes of *T. gondii* in pigs have been reported worldwide, there is no information on the genotypes and diversity of *T. gondii* in pigs in Grenada, West Indies.

Objectives: The aims of the present study were to isolate, genotype and determine the diversity of *T. gondii* genotypes in pigs.

Methods: We carried out a modified agglutination test (MAT) on blood from 149 pig hearts collected from a local meat market. Myocardial tissue homogenate from pigs that tested positive for *T. gondii* was homogenized and inoculated into mice for isolation of the parasite. We collected mouse tissues and extracted DNA for genotyping based on 11 polymerase chain reaction-restriction fragment length polymorphism markers (SAG1, SAG2, alt. SAG2, SAG 3, BTUB, GRA6, L358, PK1, C22-8, C 29-2 and Apico).

Results: Out of the 149 pig hearts, 31 (20.8%) tested positive for *T. gondii* on MAT. Bioassays in mice yielded 12 isolates designated TgpgGr1 to TgpgGr12. Molecular characterisation of *T. gondii* revealed four genotypes as follows: ToxoDB #2-clonal type III (seven isolates); ToxoDB #7 (three isolates); ToxoDB #13 (one isolate); ToxoDB #30 (1 isolate). Overall, ToxoDB #2 was the most common (58%). Toxo database (DB) # 13, which causes interstitial pneumonia in affected mice, has also been reported.

Conclusion: The genetic diversity of *T. gondii* in pigs in Grenada is lower than that in other surrounding Caribbean areas.

KEYWORDS
Genotyping, Grenada, isolation, pigs, *Toxoplasma gondii*

1 | INTRODUCTION

Toxoplasmosis is an important zoonotic disease caused by a protozoan parasite, *Toxoplasma gondii*. This parasite is capable of infecting all warm-blooded animals worldwide (Dubey, 2010). Animal intermediate hosts, such as pigs, become infected by ingesting oocysts from the environment. The ingested oocysts eventually develop into infective tissue cysts in intermediate hosts, which in turn act as a source of infection

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to definitive hosts. Felines are important in the life cycle of *T. gondii* because they are the only definitive hosts capable of shedding environmentally resistant oocysts in nature (Weiss & Kim, 2020). Humans become infected post-natally by ingesting tissue cysts from undercooked meat, consuming food or water contaminated with oocysts and accidentally ingesting oocysts from the environment (Velmurugan et al., 2008).

Historically, *T. gondii* was considered clonal with low genetic diversity and grouped into three major lineages, namely, types I, II and III, based on the analysis of 106 isolates from North America and Europe (Howe & Sibley, 1995). However, research over the past years has revealed that the genetic diversity of *T. gondii* is far greater than previously thought. The population structure of *T. gondii* is strongly subdivided by geographic region and by the existence of clonal lineages in some regions (Lehmann et al, 2006; Shwab et al., 2014; Su et al., 2012). For example, sampling from South America has revealed that strains from this region are highly divergent and comprise novel groups with both clonal and non-canonical genotypes (Khan et al., 2006; Lehmann et al., 2006; Pena et al., 2008). Importantly, some of these South American *T. gondii* lineages can cause severe ocular disease in humans (De-la-Torre et al., 2013; Khan et al., 2006). In the Caribbean countries of French Guiana, Martinique and Guadeloupe, severe disease and fatalities due to non-canonical *T. gondii* genotypes have been reported both in immunocompetent adult patients and in immunocompromised individuals (Ajzenberg et al., 2009; Carme et al., 2002; Demar et al., 2007). Therefore, this suggests that differences in clinical severity may be influenced by parasite genotype.

In Grenada, molecular characterisation of *T. gondii* based on the currently employed multiplex nested restriction fragment length polymorphism (Mn-RFLP) markers (Su et al., 2010) has only been performed in stray dogs, mongooses, rats and free-range chickens (Chikweto et al., 2017; Choudhary et al., 2013; Dubey et al., 2006, 2013). From a public health point of view, it would be more important to investigate food animals such as pigs for *T. gondii* genotypes, as infection rates in these animals can play a major role in the prevalence of the parasite in humans (Robert-Gangneux & Dardé, 2012).

Although the seroprevalence of *T. gondii* has been estimated at 23% to 24% (Chikweto et al., 2011; Sharma et al., 2014) in pigs from Grenada, to our knowledge, no attempt has been made to isolate and genotype *T. gondii* in these animals. Additionally, although outbreaks of toxoplasmosis involving non-canonical genotypes have been reported in pigs in Italy, Korea and China (Gelmetti et al., 1999; Kim et al., 2009; Li et al., 2010), no clinical toxoplasmosis has been reported in pigs in Grenada. Therefore, the aims of the present study were to isolate, genotype and determine the diversity of *T. gondii* in pigs in Grenada, West Indies.

Impacts

- We report the isolation and genotyping of *T. gondii* in pigs from Grenada, West Indies, for the first time. These findings have public health implications.
- Among the *T. gondii* genotypes identified, ToxoDB #2 (clonal type III) is the most common, albeit with limited diversity. ToxoDB #7, a strain closely related to ToxoDB #2, predominates among the non-canonical strains.
- ToxoDB #13 (Caribbean 1), a non-canonical strain, has also been reported in pigs in Grenada.

2 | MATERIALS AND METHODS

2.1 | Site description of the present study

Grenada is a small tri-island state located in the eastern Caribbean with an area of approximately 344 km^2. Grenada, the main island, is divided into six parishes. The other sister islands that are part of Grenada are Carriacou and Petit Martinique. The island has a warm and humid climate with annual temperatures ranging from 24 to 30°C (National portal of the Government of Grenada, 2015).

2.2 | Sample collection

One hundred forty-nine pig hearts were purchased from a meat market. The hearts were transported in a cooler box to the veterinary diagnostic laboratory at the School of Veterinary Medicine, St. George's University, for further processing.

2.3 | Serology and mouse bioassay

Blood from each pig heart was collected and tested for immunoglobulin G (IgG) antibodies to *T. gondii* using a modified agglutination test (MAT) as described by Dubey and Desmonts (1987), using a cut of titer of 1:25.

Myocardial samples from MAT-positive pigs were bioassayed in BALB/c mice for *T. gondii* following a previously published method (Dubey, 2010). Briefly, 30 g of myocardial tissues was homogenized in a 0.85% sodium chloride (NaCl) solution (normal saline), digested in pepsin, centrifuged at 1200 g, neutralised with sodium bicarbonate and centrifuged at 1200 g. The sediment was then suspended in 5 ml of normal saline containing 1000 units of penicillin G and 100 lg of streptomycin per ml for inoculation into two BALB/c mice. At 2 weeks post infection, the mice were bled and tested for the presence of IgG antibodies using MAT to check for seroconversion. The mice were observed for at most 2 months before euthanasia. A full necropsy was undertaken on each mouse, and brain crush smears were made to check for the presence of tissue cysts. Mice without notable IgG antibodies to *T. gondii* and/or tissue cysts were considered not infected.

The brains from chronically infected mice that showed evidence of seroconversion and/or brain cysts were subsequently homogenised and inoculated into two immunosuppressed BALB/c mice. Immunosuppression was achieved by administering dexamethasone (15 μg/ml) in drinking water for 5 days prior to inoculation (Kang et al., 2006). The

mice were monitored for *T. gondii* clinical signs for 2–3 weeks before natural death or humane euthanasia. Following a complete necropsy, impression smears from lungs, liver and peritoneal fluid were made, stained with a Romanowski stain (Diff-Quick) and examined for the presence of tachyzoites. Representative samples were also collected and fixed in 10% buffered formalin for histopathology. In addition, brain, lungs, and peritoneal fluid were harvested and stored at −80°C for DNA extraction.

2.4 | DNA extraction

DNA was extracted from the lungs, brain and peritoneal fluid of mice using a commercial kit (Bioneer Corporation) according to the manufacturer's instructions. Measurement of DNA concentration and purity was done by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) according to instructions from the manufacturer.

2.5 | Genetic characterisation of *T. gondii*

DNA derived from infected mouse tissues was used for multiplex nested polymerase chain reaction (PCR)-RFLP. Subsequent genotyping was done according to a published method (Su et al., 2010), with modifications. The genetic markers that were targeted were as follows: SAG 1; SAG2; alt. SAG2; SAG 3; BTUB; GRA6; L358; PK1; C22-8; C 29-2 and Apico as described previously (Su et al., 2010). *Toxoplasma gondii* reference strains (GT1, PTG, toxoplasma gondii type III reference strain (CTG), MAS, TgCgCa1, TgCtBr5, TgCtBr64 and TgRsCr1) were used as positive controls. Designation of *T. gondii* pig isolates to genotypes was done by referring to the *T. gondii* database at http://toxodb.org.

2.6 | Analysis of *T. gondii* isolates

We used SplitsTree version 4.17.1 software (Huson & Bryant, 2006) for construction of a phylogenetic tree. This was done to determine the relationships between *T. gondii* pig isolates in Grenada and the *T. gondii* reference strains.

3 | RESULTS

Using the MAT, IgG antibodies to *T. gondii* were detected in 31 (20.8%) out of the 149 pigs, with titers of 25 in nine pigs, 50 in 17 pigs and 100 in five pigs. However, viable *T. gondii* was isolated in mice from only 12 positive pigs, giving an isolation rate of 38.7%. The titers in these pigs were as follows: 100 in three pigs; 50 in five pigs and 25 in four pigs. These isolates were designated as TgpgGr1 to TgpgGr12 (Table 1).

In chronically infected mice, no clinical signs were observed except for a pair of mice inoculated with TgPgGr7 that died of interstitial pneumonia 2 weeks post inoculation. Gross lesions were not seen in the rest of the mice, but cytology and/or histopathology revealed tissue cysts in the brain with no evidence of inflammation. In immune-suppressed mice herein referred to as acutely infected, there was moderate to severe diffuse interstitial pneumonia.

TABLE 1 Serology and isolation of *Toxoplasma gondii* from pigs in Grenada, West Indies

Pig ID	MAT	Titer Isolate designation
P4	100	TgPgGr1
P16	25	TgPgGr2
P35	100	TgPgGr3
P41	50	TgPgGr4
P58	50	TgPgGr5
P64	50	TgPgGr6
P65	50	TgPgGr7
P72	25	TgPgGr8
P75	25	TgPgGr9
P78	25	TgPgGr10
P97	50	TgPgGr11
P108	100	TgPgGr12

Abbreviation: MAT, modified agglutination test.

PCR Mn-RFLP genotyping of *T. gondii* DNA extracted from mouse tissues based on all the 10 PCR-RFLP markers was complete on 11 isolates and partial on one isolate. Four genotypes were found, including ToxoDB #2 (clonal Type III, 7 isolates); ToxoDB #7 (three isolates); ToxoDB #13 (one isolate) and ToxoDB #30 (one isolate). Genotyping results are shown in Table 2.

Phylogenetic analysis using SplitsTree 4.17.1 software indicated that most of the *T. gondii* isolates were related to the CTG reference strain, a Toxo Db #2 (type III) genotype. The diversity among the *T. gondii* genotypes was found to be minimal, with clustering of genotypes into two main groups (Figure 1).

4 | DISCUSSION

The results of this study indicate that there is limited diversity of *T. gondii* genotypes in asymptomatic pigs in Grenada. Four genotypes were recorded, and the most common was the Toxo database (DB) #2 (type III) genotype, comprising 58% of the 12 *T. gondii* isolates. Others were non-canonical consisting mainly of Toxo DB #7, a closely related genotype to Toxo DB #2 (type III; Table 2). The observation of Toxo DB#2 as the main genotype is consistent with a previous study by Chikweto et al. (2017), which also demonstrated that this was the main genotype detected in chickens on the island. Other genotypes isolated from non-food animals are shown in Table 3.

Toxo DB #2 (type III) has also been described as the predominant genotype in pigs, goats and sheep in the neighbouring Caribbean Island of St. Kitts and Nevis (Hamilton et al., 2015). Similarly, Rajendran et al. (2012) found Toxo DB #2 and Toxo DB #7 to be the predominant genotypes in chickens in some central and southern American countries.

TABLE 2 PCR restriction fragment length polymorphism (RFLP) genotypes of *T. gondii* isolates in pigs in Grenada, West Indies

Strain designation	ToxoDB PCR-RFLP genotypes#	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												
TgPgGr1	#30	I	III	III	I	III	III	III	III	III	I	III
TgPgGr2	#7	I	III	III	III	III	III	III	III	III	III	I
TgPgGr3	#2	II or III	III	III	III	III	III	III	III	III	III	III
TgPgGr4	#2	II or III	III	III	III	III	III	III	III	III	III	III
TgPgGr5	#2	II or III	III	III	III	III	III	III	III	III	III	III
TgPgGr6	#2	II or III	III	III	III	III	III	III	III	III	III	III
TgPgGr7	#13	I	I	I	I	I	III	II	III	III	I	III
TgPgGr8	#2 likely	II or III	III	III	III	ND	III	III	III	III	ND	III
TgPgGr9	#7	I	III	III	III	III	III	III	III	III	III	I
TgPgGr10	#2	II or III	III	III	III	III	III	III	III	III	III	III
TgPgGr11	#7	I	III	III	III	III	III	III	III	III	III	I
TgPgGr12	#2	II or III	III	III	III	III	III	III	III	III	III	III

TABLE 3 *Toxoplasma gondii* genotypes in pigs, compared to the genotypes circulating in free-range chickens and non-food animals in Grenada, West Indies

Animal	Number of isolates	Toxo DB genotypes	Reference
Rat	1	**1 genotype** #2 (clonal type III)	Dubey et al. (2006)
Mongoose	4	**3 genotypes** #2 (clonal type III, one isolate); #7 (two isolates) #216 (one isolate)	Choudhary et al. (2013)
Stray dog	12	**6 genotypes** #1(one isolate); #2(clonal type III, six isolates); #3 (one isolate) #7 (two isolates) #13 (one isolate) #224 (one isolate)	Dubey et al. (2013)
Free-range chicken	20	**4 genotypes** #2(clonal type III, 15 isolates); #7 (one isolate); #13 (three isolates); #259 (one isolate)	Chikweto et al. (2017)
Pig[a]	12	**4 genotypes** #2 (clonal type III, seven isolates); #7 (three isolates); #13 (one isolate); #30 (one isolate)	

[a]Present study.

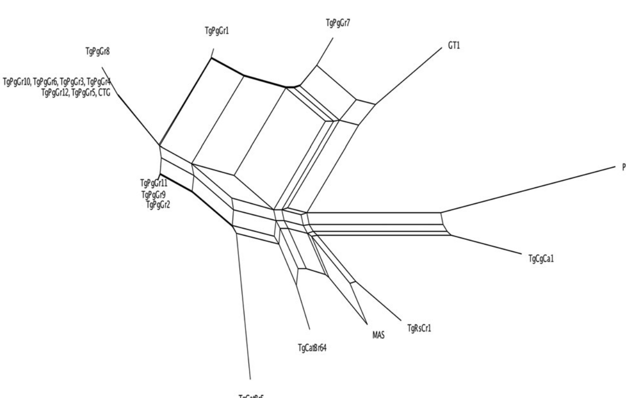

FIGURE 1 Phylogenetic analysis of *Toxoplasma gondii* isolates in pigs in Grenada, West Indies. The tree was constructed using SplitsTree4 version 4.17. 1 software. Reference strains: GT1; PTG; CTG; MAS; TgCgCa1; TgCtBr5; TgCtBr64; TgRsCr1. Grenada isolates: TgPgGr 1- TgPgGr 12

In contrast, there is a wider diversity of *T. gondii* genotypes (Hamilton et al., 2017; Hamilton, Black, et al., 2019; Hamilton, Robins, et al., 2019) in free-range chickens of the Caribbean islands of St. Kitts and Nevis; Antigua and Barbuda; Dominica and Trinidad, as most of the isolates were non-canonical.

None of the *T. gondii* genotypes isolated from pigs were lethal in infected mice except for one Toxo DB #13 isolate. Toxo DB#13 has also been reported in previous studies investigating dogs and free-range chickens in Grenada (Chikweto et al., 2017; Dubey et al., 2013). Similarly, in a recent experimental study involving several *T. gondii* isolates from Brazil, Europe and the Caribbean, ToxoDB #13 was also found to be highly lethal in mice (Hamilton, Robins, et al., 2019). Additionally, a study conducted by Hamilton et al. (2017) in St Kitts and Nevis and other islands in the Caribbean did not find lethal *T. gondii* genotypes in food animals except for ToxoDB #13 and ToxoDB #141.

This Toxo DB #13 genotype (Caribbean 1) caused severe disease and fatalities in human immunodeficiency virus (HIV) patients in Guadeloupe and Martinique (Ajzenberg et al., 2009). It is important to note that in immunocompromised people, the non-virulent genotypes, including the ones we found in the present study, can result in clinical disease and fatalities (Ajzenberg et al., 2009; Grigg et al., 2001). Hence, HIV patients in Grenada could be at risk of contracting and likely dying from the Toxo DB #13 genotype.

The prevalence of *T. gondii* in food animals is higher in pigs, sheep and goats than in cattle (Dubey, 2010) and varies worldwide (Tenter et al., 2000). Previous studies in Grenada and St. Kitts and Nevis have reported a high *T. gondii* seroprevalence in cats on the islands, indicating that the environment may be contaminated with oocysts shed in their faeces (Hamilton et al., 2014). A survey of cats in Grenada indicated that 31% of domestic cats and 28% of feral cats had antibodies to *T. gondii* (Dubey, Lappin, et al., 2009). Studies in St. Kitts and Nevis have also demonstrated 85% in domestic cats (Moura et al., 2007), and 74% of feral cats have antibodies to *T. gondii* (Dubey, Moura, et al., 2009). Furthermore, in Grenada, the seroprevalence of *T. gondii* in pregnant women ranges from 37% to 57% (Asthana et al., 2006; Dubey et al., 2016).

Toxoplasma gondii infections in pigs are mostly acquired either by ingestion of sporulated oocysts in contaminated soil, feed and water or by ingestion of cysts in the tissues of infected intermediate hosts (Stelzer et al., 2019). In conventional pig breeding systems, *T. gondii* infection through oocysts accounts for most infections (Kim et al., 2009; Li et al., 2010).

At the farm level, biosecurity measures, including the covering of food storage, neutering of cats and rodent control, have been shown to reduce the prevalence of toxoplasmosis in pigs (Eppink et al., 2021). We, therefore, recommend the same measures for pig farms in Grenada. On the other hand, the findings in the present study pose a risk for human infection with *T. gondii*. Therefore, to prevent human toxoplasmosis, persons should wash their hands thoroughly with soap and water after handling pork (Hill & Dubey, 2002). Additionally, cutting boards, sink tops, knives and utensils coming in contact with pork must also be washed with soap and water (Hill & Dubey, 2002). Another preventive measure is ensuring that meat is cooked until the internal temperature reaches 66°C before consumption (Dubey, 2010).

5 | CONCLUSION

In conclusion, we have demonstrated that Toxo Db # 2 (Type III) is the predominant genotype in pigs. *Toxoplasma gondii* in pigs highlights pork as a possible source of infection if handled with poor hygiene standards or consumed undercooked. These results are suggestive of widespread environmental contamination with *T. gondii* oocysts shed by cats. Further studies to determine *T. gondii* infection and genetic variation in humans in Grenada are required.

ACKNOWLEDGEMENT

The authors are grateful to St. George's University's small grant initiative for funding the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

Ethical approval to conduct this research was granted by the Institution of Animal Care and Use Committee (IACUC) at St. George's University (IACUC number 13016-R).

AUTHOR CONTRIBUTIONS

Conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, writing–original draft: Alfred Chikweto. *Conceptualisation, formal analysis, methodology, resources, supervision, validation, visualisation, writing–review and*

editing: Andy Alhassan. *Conceptualisation, formal analysis, methodology, resources, software, validation, writing review and editing*: Chunlei Su. *Conceptualisation, funding acquisition, methodology, project administration, resources, validation, writing–review and editing*: Calum Macpherson. *Conceptualisation, formal analysis, funding acquisition, writing–review and editing*: Muhammad Iqbal Bhaiyat. *Conceptualisation, methodology, resources, supervision, and writing–review and editing*: Jitender P. Dubey.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.870

ORCID

Alfred Chikweto https://orcid.org/0000-0002-0039-9993
Calum Macpherson https://orcid.org/0000-0002-2733-7856

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How to cite this article: Chikweto, A., Alhassan, A., Su, C., Macpherson, C., Bhaiyat, M. I., & Dubey, J. P. (2022). *Toxoplasma gondii* clonal type III is the dominant genotype identified in Grenadian pigs. *Veterinary Medicine and Science*, *8*, 2005–2011. https://doi.org/10.1002/vms3.870