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Seroprevalence and genetic characterization of *Toxoplasma gondii* in domestic pigs intended for human consumption in Cuba

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Abstract

Domestic pigs are considered as one of the main intermediate hosts in the zoonotic transmission of *Toxoplasma gondii* in many countries. Serological and molecular studies are warranted to better understand the epidemiology and transmission patterns of this parasite worldwide. To date, seroepidemiological information on *T. gondii* in domestic pigs in Cuba is very scarce and there are no reports of *T. gondii* genotypes circulating in this country. Here, we aimed to estimate the seroprevalence of *T. gondii* and provide genetic characterization of the strains circulating in slaughtered pigs intended for human consumption in Central Cuba. Seroprevalence was determined in 450 serum samples from slaughtered pigs in Villa Clara province using ELISA. Anti-*Toxoplasma gondii* IgG antibodies were detected in 100 animals (22.2%, 95% CI: 18.5–26.2). Conventional PCR of the 529-bp marker of *T. gondii* was performed in hearts and diaphragm tissues of all ELISA-seropositive pigs. *Toxoplasma gondii* DNA was detected in four animals. Further genetic characterization of the positive DNA samples was performed by multilocus PCR-RFLP and PCR-sequencing typing tools. Molecular analysis revealed four different genetic profiles that were combinations of type I, II, III and u-1 alleles, suggesting the circulation of non-clonal genotypes of

T. gondii in domestic pigs in Cuba. Our results indicate that *T. gondii* is widely distributed in slaughtered pigs in this country, which might have important implications for public health. To the best of our knowledge, this is the first report on genetic characterization of *T. gondii* in Cuba. Although preliminary, the results suggest a high genetic diversity of *T. gondii* in the study region. Further studies based on parasite isolation are needed to definitively identify the genotypes circulating and characterize the virulence of strains detected in pigs in Cuba, and to assess the risk of zoonotic transmission from pork products in this country.

KEYWORDS
Cuba, domestic pigs, genetic characterization, serosurvey, *Toxoplasma gondii*

1 | INTRODUCTION

Toxoplasmosis is an important zoonotic disease of worldwide distribution. It is caused by the protozoan parasite *Toxoplasma gondii*, which infects most homeothermic animals, including humans (Dubey, 2022). Approximately 30% of the world's human population is infected with this intracellular obligate parasite (McLeod et al., 2020). Although the infection by *T. gondii* is typically asymptomatic in immunocompetent individuals, the parasite can cause neurological and ophthalmological diseases when acquired postnatally, and serious, even fatal, cases in children infected congenitally (McLeod et al., 2020). Moreover, accumulating evidence suggests that latent infection with *T. gondii* might be associated with a variety of neuropsychiatric and behavioural conditions in human beings (Milne et al., 2020).

Consumption of infected raw or undercooked meat from farm animals, mostly pork, is considered the main route of *T. gondii* infection in humans in many countries (Belluco et al., 2018; Dubey et al., 2020). Pork is the most frequently consumed meat in Cuba (ONEI, 2017); however, epidemiological studies on *T. gondii* in pigs are very scarce in the country (Castillo-Cuenca et al., 2021; Suárez-Hernández et al., 2005), and survey studies in pigs intended for human consumption have not been carried out to date. Despite sources of infection not being attributed, clinical cases, including ocular toxoplasmosis in immunocompetent people, have been reported in this country (Bustillo et al., 2015; Ginorio Gavito et al., 2017). The incidence of congenital toxoplasmosis is unknown.

Currently, the *T. gondii* population is considered complex and diverse, constituted by 16 haplogroups assorted into six major clades (Lorenzi et al., 2016); indeed, more than 300 distinct genotypes have been identified (ToxoDB.org). Studies in the Caribbean area reported a high frequency of non-canonical strains and a noticeable proportion of type III strains (Dubey et al., 2016). Although congenital toxoplasmosis has been reported in humans in Cuba affecting different organs (Amador Morán et al., 2016), to our knowledge, there is no previous research on genetic characterization of *T. gondii* in Cuba. The main aims of the present study were to determine the seroprevalence of *T. gondii* in slaughtered pigs intended for human consumption and to genetically characterize *T. gondii* strains infecting such host species in Cuba.

Impacts

- *Toxoplasma gondii* is widely distributed in slaughtered pigs intended for human consumption in Cuba.
- First report on genetic characterization of *T. gondii* in Cuba.
- *T. gondii* DNA samples characterized presented genetic profiles related to non-canonical genotypes.
- Molecular results suggest a high genetic diversity of *T. gondii* in the study region.
- Control measures should be implemented to reduce the risk of exposure to *T. gondii* in pigs in Cuba.

2 | MATERIAL AND METHODS

2.1 | Study area and sampling

A cross-sectional study was performed to determine the individual seroprevalence of *Toxoplasma gondii* IgG antibodies and assess the genotypes circulating in fattening pigs intended for human consumption in Villa Clara (largest pork producer province in Cuba; Figure 1).

The total pig population of all fattening farms in the study area (>10.000) was used to calculate sampling size. The sample size was established assuming an estimated *T. gondii* seroprevalence of 50%, which provides the highest sample size in studies with unknown prevalence (Thrusfield et al., 2018), a desired absolute precision of ±5%, and a 95% confidence level (95%CI), resulting in 384 domestic pigs being sampled.

A total of 450 blood samples, paired with heart and diaphragm tissues, were finally collected from selected slaughtered pigs (animals

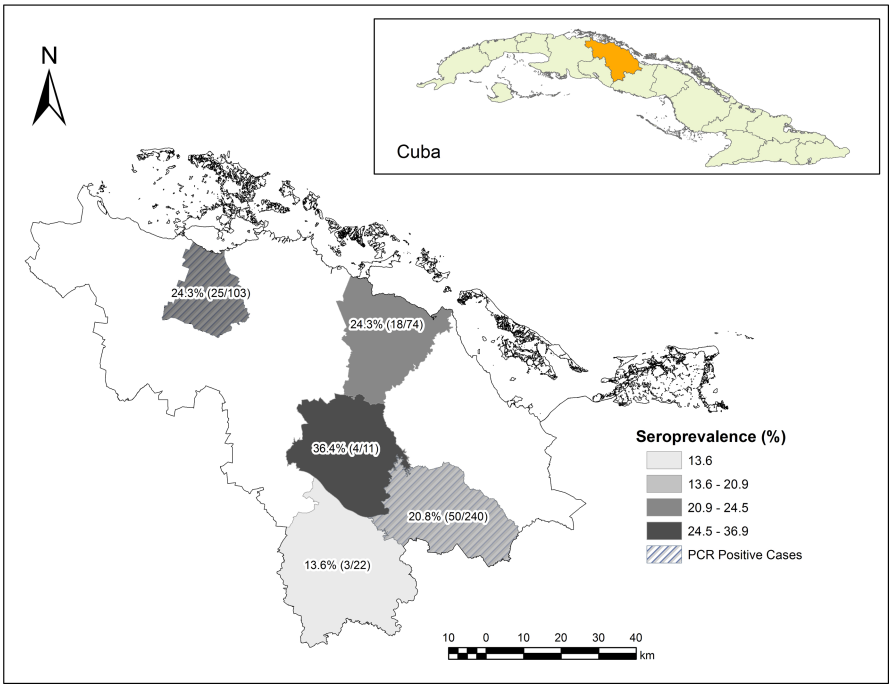

FIGURE 1 Spatial distribution of *Toxoplasma gondii* seropositivity in slaughtered pigs in Villa Clara province (Central Cuba)

of around 6 months of age and 90 kg of weight on average) intended for human consumption at the main slaughterhouse in the province of Villa Clara. All the pigs slaughtered and sampled came from private farms, a distinct characteristic of pig fattening in Cuba (Castillo-Cuenca et al., 2021). Samples were collected from January to March 2019 during nine sampling days, taking 50 samples per sampling day. Animals were selected by a systematic random sampling method. For each sampled pig, at least 100 g of diaphragm and heart were collected, introduced in individual plastic bags, refrigerated at 4°C during transportation to the laboratory and frozen at −20°C until analysis. Moreover, approximately 10 ml of blood was collected at the time of bleeding in the slaughter line. Data on sex and origin (at municipality level) were recorded for each sampled pig.

2.2 | Serological analysis

Blood samples were centrifuged at 400×*g* for 10 min. Serum was separated and stored at −20°C until tested. Serum samples were analysed using a commercial indirect enzyme linked immunosorbent assay (ELISA; PrioCHECK® *Toxoplasma* Ab porcine, Thermo Fisher Scientific Prionics AG) in accordance with the manufacturer's recommendations as previously described (Castillo-Cuenca et al., 2021). The sensitivity and specificity rates of this ELISA provided by the manufacturer were 98.0% and 99.6%, respectively.

2.3 | Genomic DNA extraction

Genomic DNA was extracted from each tissue sample using the commercial QIAamp® Fast DNA Tissue kit (Qiagen®) according to the manufacturer's recommendations. Briefly, 25 mg of each tissue were incubated with proteinase K at 56°C for 10 min in a thermomixer. DNA samples were obtained after purification by silica gel column chromatography and eluted into 100 µl of elution buffer.

2.4 | DNA detection

Screening for the *T. gondii* DNA presence in the DNA samples extracted from pig tissues was carried out by a conventional PCR amplification of the specific 529-bp repetitive element marker (Homan et al., 2000). In brief, the PCR reaction was performed in a 25 µl using HotStar Plus DNA Polymerase and a Bio-Rad T100 thermocycler. PCR products were evaluated on 2% agarose gels in 0.5X TBE buffer, stained with ethidium bromide and visualized under UV light.

2.5 | Genetic characterization by multilocus nested-PCR-RFLP

Toxoplasma gondii strains detected were characterized by a multilocus nested (Mn)-PCR-RFLP method based on ten genetic markers *SAG1*, *SAG2* (*3′-SAG2* and *5′-SAG2*), *SAG3*, *GRA6*, *C22-8*, *L358*, *BTUB*, *C29-2*, *PK1* and *Apico* (Su et al., 2010). Basically, the multiplex PCR reaction was carried out in a 50 µl reaction volume containing 0.15 µM of each of the external forward and reverse primers, 200 µM of dNTPs (Eurogentec, Belgium), 2 mM of $MgCl_2$, 1X Q solution, 1X PCR Buffer, 1 Unit HotStart Plus DNA Polymerase and 10 µl of sample DNA (Qiagen®). As positive controls, 2 µl DNA extracted from the strains RH (Type I, ToxoDB#10), Wil (Type II, haplogroup 2), and PRU (Type II variant, ToxoDB#3) was used. Nuclease-free water was used as negative control. The reaction mixture was treated at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min.

Subsequent individual nested PCR (nPCR) reactions were carried out in 25 µl volume containing 0.3 µM of each corresponding pair of internal forward and reverse primers, 200 µM of dNTPs, 2 mM of $MgCl_2$, 1X of Q solution, 1X of PCR Buffer, 1 Unit of HotStart Plus DNA Polymerase, and 4 µl of previous multiplex PCR product. PCR products from internal reactions were analysed by 2% agarose gel electrophoresis, stained with ethidium bromide, and examined under UV light. Each positive nPCR product (7 µl) was digested with restriction endonucleases in a 10 µl volume then resolved on a 3% agarose gel to reveal the DNA banding pattern. The DNA restriction patterns obtained were compared with the profiles deposited in ToxoDB (http://toxodb.org/toxo/).

2.6 | Genetic characterization by PCR-sequencing

Because of the interest in sequence-based genotyping (Fernández-Escobar et al., 2020) and aiming to phylogenetic analyses, the amplicons of the *SAG1* and *SAG3* markers were further sequenced using a 3730XL automatic sequencer from Applied Biosystems (ABI; Microsynth Seqlab, Maschmühlenweg). These sequences were curated manually when necessary and analysed using BioEdit software, version 7.0.5.3 (Hall, 1999). *SAG1* and *SAG3* sequences were also concatenated and aligned using MEGA X software (Kumar et al., 2018) to generate an unrooted phylogenetic tree and evaluate the population structure of *T. gondii* Cuban strains described here; type I and II reference strains (RH, Tg51, Wil and PRU) were included for comparison. On the other hand, generated *SAG3* sequences were also aligned to similar available sequences originated in America retrieved from the NCBI database through the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using MEGA X software (Kumar et al., 2018). The evolutionary history was inferred by the Neighbour-Joining method (Saitou & Nei, 1987) and conducted in MEGA X software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches in each tree (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). The sequences obtained in the present study have been deposited in GenBank (*SAG3*: OM836491-OM836494; *SAG1*: OM648096- OM648099).

2.7 | Statistical analyses

Individual seroprevalence was estimated from the ratio of ELISA-positive sera to the total number of analysed samples, with 95% exact binomial confidence intervals (95% CI). Associations between serological results and independent variables (sex and municipality) were analysed by Pearson's chi-square or Fisher's exact tests, as appropriate. Differences were considered statistically significant when p-value <.05. Statistical analyses were performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA).

3 | RESULTS

One hundred of the 450 sera were positive for anti- *Toxoplasma gondii* IgG antibodies (22.2%; CI 95%: 18.5–26.2). Seropositive pigs were detected in all the municipalities sampled, and the within-municipality seroprevalence ranged between 13.6% and 36.8% (Figure 1). Statistically significant differences in seroprevalence between sexes (22.2% [54/243] and 22.2% [46/207] in males and females, respectively) (p = .545) or municipalities (p = .572) were not found.

Toxoplasma gondii DNA was detected in heart samples from four of the 100 ELISA-seropositive individuals (4%; pigs 77, 97, 129 and 143) by conventional PCR. *Toxoplasma gondii* DNA was also found in diaphragm tissues from pig 97 (1%). PCR-positive pigs were two males (pigs 77 and 97) from municipality of Placetas, and one female (pig 129) and one male (pig 143) from Quemado de Güines.

Only the four *T. gondii* DNA-positive samples from heart could be successfully characterized for at least five genetic markers (SAG1, SAG2, SAG3, c22-8 and L358); samples from pig 77 could be also genotyped at *GRA6* marker. Genetic characterization by Mn-PCR-RFLP revealed four different genetic profiles that were combinations of type I, II, III and u-1 alleles. Pigs 97 and 129 were shown to be infected by undescribed potentially non-canonical genotypes, the other two PCR-positive animals resulted in allele combinations that could be related to ToxoDB #138 (pig 143) or other recombinant genotypes (pig 77) (Table 1).

TABLE 1 Multilocus PCR-RFLP genotyping of *Toxoplasma gondii* DNA from domestic pigs intended for human consumption in Cuba

Pig ID	Municipality	SAG1	3′-5′SAG2	SAG3	GRA6	C22-8	L358	ToxoDB genotypes interpretation
77	Placetas	u-1	II	III	II	II	II	Six markers matched with ToxoDB #9, #20, #137
97	Placetas	u-1	III	III	Na	II	II	No match (potential undescribed genotype)
129	Quemado de Güines	I	III	I	Na	II	II	No match (potential undescribed genotype)
143	Quemado de Güines	u-1	III	III	na	III	III	Five markers matched with ToxoDB #138

Abbreviation: na: no amplification.

Regarding PCR-sequencing results, data obtained supported a certain degree of heterogenicity within the *T. gondii* population infecting pigs in Cuba. Concatenation of *SAG1*+*SAG3* markers (Figure 2a) allowed us to observe how Cuban pig samples split into two branches; sample 129 clearly clustered with RH strain, in agreement with its type I-like RFLP profile, whereas samples 77, 97 and 143 clustered together conditioned by the allele u-1 present in *SAG1* marker. On the other hand, the detected SNPs G1593T, G1594A and A1614G allowed the discrimination in a separate branch of the sample 143. G1593T and G1594A lead to an amino acid change from Gly to Tyr, while A1614G causes a change from Ser to Gly.

Phylogenetic analyses based on *SAG3* marker sequences obtained here in addition to those retrieved from NCBI related to strains originated in America (North, Central, and South American territories) are shown in Figure 2b. The tree showed a clear clustering of samples from pigs 77 (GenBank Accession Number: OM836491) and 97 (OM836492). Sample from animal 143 (OM836494) showed 100% homology with the isolate MH744787 from American black bear (*Ursus americanus*), among others. Finally, sample from pig 129 (OM836493) clustered with type I-related strains. In addition, this sample grouped close to sequences MK127861, MH744785, and MK875303 of recombinant isolates which have been identified as atypical variants at the *SAG3* region.

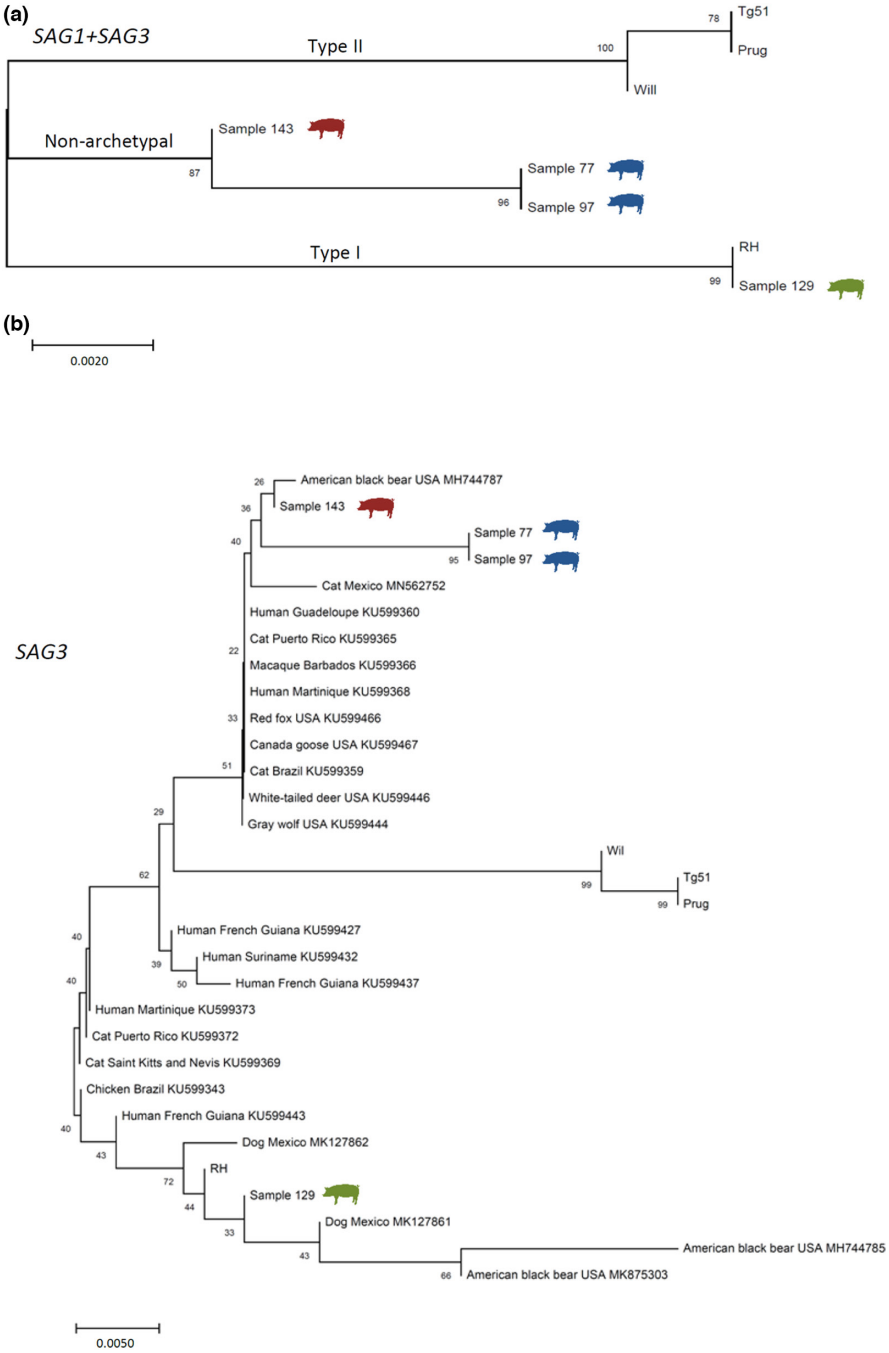

FIGURE 2 Phylogenetic positioning of the *Toxoplasma gondii* genetic variants detected in domestic pigs from Cuba based on (a) concatenated *SAG1*+*SAG3* sequences generated in this study, and (b) *SAG3* sequences from Cuban pigs along with similar available sequences originated in America (North, Central, and South American territories) retrieved from the NCBI database. The evolutionary history was inferred using the neighbour-joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed by the maximum composite likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018)

4 | DISCUSSION

Public concerns associated with *Toxoplasma gondii* clearly indicate the need for epidemiological investigation in animals used as a source of food. In this regard, the consumption of raw or undercooked pork products is considered as one of the main sources of *T. gondii* infections in humans in many countries (Dubey et al., 2020). In the present study, we provide epidemiological information on *T. gondii* in slaughtered pigs in Cuba, a country where a very limited information on this zoonotic protozoan has been reported in livestock to date.

To the best of the authors' knowledge, this is the first survey on *T. gondii* conducted in fattening pigs intended for human consumption and the first genetic characterization study on this parasite in this country. The seroprevalence obtained (22.2%) is of the same magnitude than those reported globally (19%) and for Central and South America (23%) (reviewed by Foroutan et al., 2019). Higher mean seroprevalence values in pigs (ranging between 32.5% and 96.6%) have been observed in other Central and South American countries (reviewed by Dubey, 2022), while lower seroprevalence values have been detected in fattening pigs raised in intensive farms in Brazil (ranging between 0% and 8%) (Frazão-Teixeira & de Oliveira, 2011; Miura et al., 2019; Oliveira et al., 2019), Chile (9%) (Muñoz-Zanzi et al., 2012) and Mexico (ranging between 1% and 9%) (Alvarado-Esquivel et al., 2012; Cubas-Atienzar et al., 2019).

To date, only two previous studies have been carried out on seroprevalence of *T. gondii* in domestic (not fattening) pigs in Cuba. In a first study, conducted in 2189 pigs sampled in Ciego de Ávila province (Central Cuba) during the period 1980–2002, Suárez-Hernández et al. (2005) reported a seroprevalence of 14%. More recently, similar overall seroprevalence (13%) was found in sows and post-weaning piglets in the province of Villa Clara (Castillo-Cuenca et al., 2021). Of note, the seroprevalence detected in fattening pigs intended for human consumption in our study (22.2%) was very similar to that found in sows (21.9%) and considerably higher than the observed in post-weaning piglets (4.8%) in that previous study. The higher *T. gondii* seropositivity observed in sows and slaughtered pigs compared with the post-weaning period in Cuban pigs agrees with those previously reported (Dubey et al., 2020; García-Bocanegra et al., 2010) and reflects a cumulative probability of exposure to *T. gondii* and lifelong persistence of antibodies. In addition, the higher *T. gondii* exposure during the finishing period could be due to the more hygienically relaxed management conditions in this period and/or the presence of outdoor facilities in growing-finishing units in the herds.

Seropositive animals were detected in all the municipalities sampled, with a within-municipality seroprevalence ranging between 13.6% and 36.9%. These findings indicate that *T. gondii* is widely distributed in slaughtered pigs from private farms in Cuba, which is in line with those previously observed in sows from breeding farms in this country (Castillo-Cuenca et al., 2021). Further studies are warranted to identify and remove the sources of exposure to *T. gondii* in pigs during the finishing period in order to reduce the zoonotic risk in Cuba.

Previous studies have shown that serological analysis of *T. gondii* using ELISA is a good method for the surveillance of the parasite in pigs (Garcia et al., 2008; Hill et al., 2006). In the present study, only four (4.0%) of the 100 ELISA-positive pigs tested positive using PCR. This finding is in accordance with those observed by Hill et al. (2006), who reported differences in the diagnostic technique sensitivities in the analysis of *T. gondii* infection in samples from both experimentally and naturally infected pigs and retail pork products, and it is due to the random distribution of tissue cysts, the limited volume of sample analysed, and the usual low parasite burden observed in the tissues of chronically infected animals. Of note, *T. gondii* DNA was more frequently detected in heart than in diaphragm tissues. This finding agrees with those reported by other authors (Gisbert Algaba et al., 2018; Vergara et al., 2018), pointing out heart as the target organ for detection of *T. gondii* infection in pigs.

Genetic characterization of *T. gondii* is crucial since the genotype can determine the presentation and severity of clinical toxoplasmosis in immunocompetent hosts (Dardé et al., 2020). Here, we attempted to molecularly characterize for the first time the *T. gondii* strains that circulate in domestic pigs in Cuba. The preliminary results suggest the existence of a high genetic diversity of *T. gondii* in slaughtered pigs in this country (Table 1; Figure 2). All four characterized samples presented genetic profiles related to non-canonical genotypes. Till date, numerous studies have confirmed the high genetic and genotypic diversity among *T. gondii* strains circulating in Central and South America, whose population structure have shown to be heterogeneous in this region (Dardé et al., 2020; Rajendran et al., 2012).

Molecular data on *T. gondii* in Central America and the Caribbean territories are still scarce. Previous studies showed that type III clonal genotype (ToxoDB#2) is common and widespread in this region and has been identified in different domestic hosts, including dogs, cats and chickens (Chikweto et al., 2017; Dubey et al., 2009, 2016; Rajendran et al., 2012), but it was not observed in the present study. To the best of our knowledge, the only available data collected from pigs in the Caribbean area were reported by Hamilton et al. (2015) in Saint Kitts and Nevis. The authors attempted direct genotyping by Mn-PCR-RFLP using four markers (*SAG2*, *SAG3*, *BTUB*, and *GRA6*) in tissue samples, and the resulting patterns suggested the presence of clonal type I (ToxoDB#10) and type III (#2) genotypes. Worldwide compilation of PCR-RFLP data (Shwab et al., 2014) identified certain geographical patterns but there are still many gaps in the Caribbean area. The analysis by microsatellite typing tools might help in this analysis, since a notorious divergence of the Caribbean organisms is expected when compared with other European or American isolates/data (Lehmann et al., 2006).

Regarding PCR-sequencing results, data obtained suggest a high degree of heterogeneity of the *T. gondii* population infecting slaughtered pigs in Cuba. Our results provided additional evidence of a rich genetic diversity of *T. gondii* in the Caribbean area. Despite its short length, *SAG3* is considered a suitable marker for phylogenetic analyses (Bertranpetit et al., 2017; Fernández-Escobar et al., 2020). In Figure 2b, taking into consideration

available (NCBI) *SAG3* sequences from livestock, wild animals and humans in the Americas, it is showed a clear cluster of the samples from two of the PCR-positive animals (pigs 77 and 97, both of them originated in the same municipality), supporting the hypothesis of a new genetic variant, presumably a new genotype infecting pigs in Cuba. On the other hand, sample from pig 143 showed 100% homology with the isolate MH744787 from American black bear (*U. americanus*), among others. The specific branch clustering Wil, Tg51 and PRU reference strains demonstrated the low occurrence of type II-related strains in general in the Americas (as opposite to Europe Fernández-Escobar et al., 2022) and specifically in the area of study. Furthermore, sample from animal 129 clustered along with type I-related strains and therefore could be presumably a virulent mice isolate; in addition, this sample grouped close to sequences MK127861, MH744785 and MK875303 of recombinant isolates showing atypical variants at *SAG3* region (Scimeca et al., 2020; Valenzuela-Moreno et al., 2020).

There is a growing body of evidence that some non-archetypal genotypes are linked to an increased occurrence of congenital and ocular toxoplasmosis in immunocompetent human patients (Dardé et al., 2020). In Cuba, unfortunately, no studies have linked clinical cases of toxoplasmosis in humans with *T. gondii* genetic characterization results. However, we hypothesized that the circulation of atypical genotypes in domestic pigs in this country could explain the high incidence rate of ocular toxoplasmosis in Cuba. In this respect, Bustillo et al. (2015) reported an incidence rate of active ocular toxoplasmosis of 58.5 per 100,000 inhabitants in the neighbouring province of Sanctis Spiritus, the highest incidence rate reported to date in a high-risk tropical region. Based on our results, further molecular studies are required to analyse the genetic population of *T. gondii* in pigs and humans in Cuba and try to unravel questions that arose here: (i) are canonical types occurring in the area and in which proportion?, (ii) are new genotypes circulating in pigs from Cuba?, and finally, (iii) how divergent is the population structure of *T. gondii* of Cuba in comparison with other Caribbean and Centro-American territories? The above questions open an important avenue to hypothesize whether the observed non-canonical strains show virulent phenotypes and to what extent they may pose a risk for human beings.

In conclusion, the results obtained in the present study indicate a wide *T. gondii* circulation in domestic pig populations intended for human consumption in Central Cuba, which is of public health concern. To the best of our knowledge, this is the first report on genetic characterization of *T. gondii* in this country. Although preliminary, the results suggest that the genetic diversity of *T. gondii* in this region is high. Further studies based on parasite isolation are needed to have a complete identification of genotypes circulating, to characterize the virulence of strains detected in pigs in Cuba, and to assess the risk of zoonotic transmission from pork products in this country.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The collection of samples analysed in the present study was part of the official Animal Health Campaigns under Cuban legislation. No animals were specifically sampled for this study; therefore, no ethical approval was necessary.

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