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1 Genotyping of *Toxoplasma gondii* from pigs in Yucatan, Mexico

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10 Abstract:

11 Toxoplasmosis is a zoonotic disease of worldwide distribution. The parasite exhibits strong geographical
12 patterns of strain variation with contrasting high levels of diversity across South America and restricted
13 variation across North America. Little is known about the diversity of strains in the transitional area between
14 the two continents. Here we present data on the prevalence and diversity of *Toxoplasma gondii* in the
15 Yucatan peninsula of Mexico, through a study in commercially reared pigs. A survey of 12 farms found
16 evidence of circulating *T.gondii* DNA in 125 of 632 blood samples (19.8%, CI: 16.7%-23%). In addition, 46
17 tongue samples were collected from culled animals and 16 of these were positive for *T. gondii* DNA and 3
18 were positive in mouse bioassay. PCR-sequencing was used to generate genotyping data from blood and
19 tissue samples. Four loci (SAG1, 2, 3 & GRA6) were reliably amplified and revealed a high diversity among
20 Yucatan strains with evidence of recombination and novel alleles. Sequencing data from the four loci was
21 achieved in eight samples each of which had a different genotype. The predominant allelic type was atypical,
22 in relation to the dominant strain types (I, II, III), the number of allelic variants being 27 (I, II-III, u-1-25), 20
23 (I, III, u1-18), 6 (I, III, u1-4) and 11 (I, II, u1-9) for the SAG1, SAG2, SAG3 and GRA6 loci respectively.
24 Phylogenetic analysis showed that *T. gondii* strains from Yucatan shared alleles with strains originating from
25 both North and South America. Our findings are consistent with data from other regions of Central America
26 and suggest the genetic population structure of the parasite, with significant levels of allelic variation and
27 recombination, constitutes a reservoir from which new strains may emerge. Positive bioassay results (7.5%)
28 indicate that consumption of undercooked pork could be a potential *T. gondii* infection risk to humans.

30 **Keywords:** *Toxoplasma gondii*, *Sus scrofa*, Mexico, isolation, Multi Locus Sequence Typing

31 **1. Introduction**

32 *Toxoplasma gondii* is an Apicomplexan parasite of worldwide distribution which can infect nearly all warm-
33 blooded vertebrates. Humans are infected through ingestion of sporulated oocysts, which contaminate the
34 environment, or from undercooked meat that contains tissue cysts. The parasite is genetically diverse and is
35 currently classified into 16 haplogroups which show clear geographical patterns of distribution with the
36 dominant presence of a few clonal genotypes in Europe and North America (Lorenzi *et al.*, 2016). In North
37 America most isolates fall into haplogroup 2 and 3 together with some presence of strains from haplogroup 1
38 and the recently described haplogroup 12. The southern continent has a very different population structure
39 represented by haplogroups 4, 5, 8, 9, 10 and 15 with highly diverse genotypes characterised by many novel
40 alleles inherited in new combinations which are not found in other regions of the world (Frazão-Teixeira *et*
41 *al.*, 2011, Rajendran *et al.*, 2012, Dubey and Su, 2009, Lehmann *et al.*, 2006, Pena *et al.*, 2008).

42 It is interesting to question how this striking variation in parasite diversity is maintained by investigating the
43 boundary between the two continents to see whether diverse southern haplotypes are present and to what
44 extent gene flow occurs. The Yucatan peninsula, located in the south of Mexico, was selected for the present
45 study because it represents part of this interface between the North and South continents.

46 A few studies have investigated the genetic diversity of the parasite in Mexico but have been mainly
47 focussed on the northern part of the country. In patients with congenital toxoplasmosis in Mexico state,
48 clinical samples from four mother-child pairs were genotyped by Restriction Fragment Length
49 Polymorphism (RFLP) using the four loci SAG2, SAG3, GRA6, BTUB and only type I genotypes with
50 unique alleles were found (Rico-Torres *et al.*, 2012). Dubey *et al.*, 2009 obtained 5 genotypes of thirteen *T.*
51 *gondii* isolates from dogs, cats and chickens in Durango (Dubey *et al.*, 2009). Four of these isolates were
52 clonal Type III and 9 had genotypes with mixed alleles. A total of two 2 isolates were recovered from wild
53 animals in Durango, one from a puma and one from a pigeon (Dubey *et al.*, 2013, Alvarado-Esquivel *et al.*,
54 2011). The isolate obtained from the pigeon had a genotype reported before in (Dubey *et al.*, 2009) obtained
55 from a cat in Durango. The isolate obtained from the puma had a novel genotype with mixed I, II and u-1
56 alleles. Rico-Torres *et al.*, (2015), identified another *T. gondii* genotype obtained from a cat in Colima also

57 with mixed I, II and III alleles. Studies in Durango and Colima used RFLP with a panel of 12 loci (Su *et al.*,
58 2010) as a genotyping technique.

59 Overall, studies in Mexico have found a predominance of the clonal Type III lineage, recombinant and
60 atypical strains with mixed I, II, III and u-1 alleles using multi locus RFLP typing. However, due to the
61 scarce data and the restricted resolution of RFLP, the question remains as to whether strains in Mexico are
62 more related to North or South America and as to which haplogroups they belong. In the current study, we
63 investigated the diversity of *Toxoplasma gondii* in the southern Mexican state of Yucatan using the highly
64 discriminative Multilocus Sequence Typing (MLST) technique.

65 The seroprevalence of *T. gondii* among the human population of Yucatan is high (70%) according to the last
66 national survey (Caballero-Ortega *et al.*, 2012). Pork is the most highly consumed meat in Yucatan as an
67 integral part of the culinary culture (Arroyo *et al.*, 1999, Ponce, 2004) and has been shown to be infected
68 with the parasite via PCR (Hernández-Cortazar *et al.*, 2016). PCR is widely used in parasite detection as it is
69 highly sensitive and allows genotyping directly from tissues (Aspinall *et al.*, 2002, Yu *et al.*, 2013). PCR
70 techniques have achieved a detection threshold down to less than one single microorganism (Lin *et al.*, 2012,
71 Jones *et al.*, 2000). Nevertheless, assessment of infection risk is best achieved through bioassay as this can
72 assess the viability of the parasite (Redondo *et al.*, 1999).

73 Our study focused on the PCR detection and genotyping of strains circulating among pigs and sympatric
74 animal species through a cross-sectional survey of commercial pig units, together with *post-mortem*
75 sampling at abattoirs. We further investigated the viability of the parasite in tissue samples by mouse
76 bioassay.

77 **2. Materials and methods**

78 In accordance with ethical considerations, the project was approved by the ethics panel of the University of
79 Salford with the reference number CST 13/72.

80 *2.1. Origin of the samples*

81 Samples were collected from 2013 to 2015 during summer seasons (June-September). Five hundred and
82 eighty-six porcine blood samples were collected from pigs raised in 12 intensive farms. In addition to this
83 cross-sectional study, 40 pig tongues and blood samples were collected from market-age pigs slaughtered in

84 two abattoirs. Pigs slaughtered at the abattoirs were destined for human consumption. Blood and tongue
85 samples were also collected from six 16-17 week old pigs euthanised in one of the farms (farm A) due to
86 poor growth and respiratory problems. One cat from the same farm was also culled by the farm veterinarian
87 as part of a measure to control the high population of cats and its brain and heart were collected for bioassay.
88 In addition to the pig sampling, in June of 2015, forty Sherman traps (HB Sherman Traps Inc., Florida, USA)
89 were placed during one week on one farm (farm B) infested with rodents. Traps were placed in the
90 warehouse, the worker's break room and across all pen areas (maternity, farrowing, weaning and fattening
91 areas). A mixture of oats with vanilla (Panti-May *et al.*, 2012) was first used but this bait was replaced with
92 pig food after observing rodent preference. Captured animals were transported to the Zoology laboratory of
93 the Faculty of Veterinary at the Autonomous University of Yucatan where they were euthanised with
94 pentobarbital (Pisabental®). The age of the rodents was calculated based on weight (Sridhara and
95 Krishnamurthy, 1992). Brain, leg muscle tissue and heart tissues were collected from all trapped rodents.

96 2.2. Viability of *T. gondii*

97 Porcine tongues and feline brain and heart tissue were processed and digested with pepsin according to a
98 protocol by Dubey (1998). Following the pepsin digestion, the sediment was mixed with 5 ml of saline that
99 contained 1000 IU of penicillin and 100 µg/ml of streptomycin and 0.5 ml of this solution was inoculated
100 intraperitoneally into 2-4 BALB/c mice using a 27G needle. Mice were individually marked by ear cutting
101 and screened for *T. gondii* infection after 2 months p.i. (Dubey, 2010). *T. gondii* diagnosis was confirmed by
102 the demonstration of the parasite in mouse brain by nested PCR amplification of the major surface antigen
103 (SAG1N-PCR).

104 2.3. DNA extraction, SAG1 N-PCR screening and genotyping

105 Porcine blood was screened by SAG1 N-PCR to assess the level of acute infection among farmed pigs. To
106 increase the sensitivity of *T. gondii* detection in blood, DNA was extracted from the leukocyte layer
107 (Brenier-Pinchart *et al.*, 2015). To isolate the leukocyte fraction, uncoagulated blood was centrifuged at
108 1300g for 30 minutes. The fine white layer, corresponding to the leukocytes, was removed carefully and
109 placed in sterile 2 ml microcentrifuge tubes. The erythrocytes remaining in the leukocyte fraction were lysed
110 according to Gallardo and Pelayo (2013). The final pellet of leukocytes was washed twice and resuspended

111 in 200 µl of phosphate buffered saline (PBS) for DNA extraction. Tissue DNA was extracted by dissecting
112 30-50 mg of the specific organ and in the case of porcine tongues, DNA was also extracted from the pellet of
113 the digested homogenate using the same weighing portion.

114 DNA was extracted with the Qiagen DNeasy Blood and Tissue Kit following the manufacturer instructions.
115 DNA concentration and purity were measured by spectrophotometry (Nanodrop 1000).

116 The diagnosis of *T. gondii* in blood and tissues was carried out using SAG1N-PCR (Su *et al.*, 2010).
117 Seventy-seven SAG1 PCR products obtained from rat tissues, mouse brain, pig tissues and blood were
118 prepared for sequencing. PCR products were purified using the kit Wizard® Gel and PCR clean-up system
119 (Promega) and sent to the company Source Biosciences where samples were processed for Sanger
120 sequencing. Both forward and reverse strands were sequenced for all samples.

121 Due to the high number of swine blood samples, only pigs raised in 6 of the 12 farms were used for
122 genotyping purposes.

123 Successfully sequenced samples, for the SAG1 marker, were then amplified with SAG2 and GRA6 primers
124 and any sample which had amplified with more than one genetic marker was then tested with SAG3.
125 Samples which had amplified with these four genetic markers were tested with additional probes (3' SAG2,
126 5'SAG2, BTUB, PK1, L358, C22-8, C29-2, Apico, UPRT1, UPRT7, EF1 and HP2) described in Su *et al.*,
127 (2010) and Su *et al.*, (2012).

128 The amplification and reaction conditions were performed as described elsewhere (Su *et al.*, 2010, Su *et al.*,
129 2012) with modifications to increase the sensitivity. The optimised external amplification was performed in a
130 volume of 25 µl with 1.25 units of Hot Start Plus Taq Polymerase (HSPT) (Qiagen), 2 µl of DNA, 2 mM of
131 MgCl₂, 200 µM of each dNTP and 0.35 µM of each external primer. The nested and semi-nested reaction
132 was carried out in a volume of 25 µl with 1.25 units of HSPT, 2 µl of the PCR product obtained in the first
133 round, 2 mM of MgCl₂, 200 µM of each dNTP and 0.2 µM of each internal primer and conditions as in Su *et*
134 *al.*, 2010. This amplification protocol was used as this had the highest sensitivity in our internal calibration
135 reaching the detection limit of ~5.7-7.1 and 14.3 parasites per reaction in a high density of host DNA (100 ng
136 of MDBK cells free from *T. gondii* DNA) for SAG1 and SAG2 markers. PCR-water (Qiagen) was used as a
137 negative control in both rounds of the N-PCR and 100 ng/µl of MDBK cells spiked with ~10-100pg of *T.*
138 *gondii* RH strain DNA was used as a positive control. To avoid cross-contamination, reagents and DNA
139 were stored in small aliquots and filter tips were used in every step. PCR products were manipulated in a

140 separate room from the PCR set up room. PCR amplifications were visualised with GelRed™ (Biotium)
141 staining on a 1% TBE (Tris-borate-EDTA) gel with 1% to 2% of agarose (Bioline) depending on the
142 fragment size to resolve and processed for sequencing as described before.

143 2.4. Data analysis

144 Statistical evaluations were performed with the data packages Epi-info (v. 7.1.3) and SPSS (v.19). DNA
145 sequences were aligned by ClustalW using default parameters in MEGA 6.06 software (Tamura *et al.*, 2013).
146 Phylogenetic trees were constructed using the Neighbour-Joining (NJ) and Unweighted Pair Group Method
147 with Arithmetic Mean (UPGMA) methods also using the default parameters in MEGA 6.06 (Pairwise
148 deletion, including transitions and transversions, uniform rates and Maximum Composite Likelihood Method
149 to calculate evolutionary distance). *T. gondii* reference sequences were downloaded from ToxoDB
150 (<http://toxodb.org/toxo/>) NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and compared using
151 BLAST.

152 3. Results

153 3.1. Detection of *T. gondii* using SAG1 N-PCR

154 The overall number of pigs (n= 632) which tested PCR positive in blood samples was 125 (19.8%, 95% CI:
155 16.7%-23%). PCR prevalence was analysed by age, gender, farm and environment, but showed no
156 significant relationship (data not shown).

157 Tongues were sampled from 46 animals slaughtered at farm A (n= 6), abattoir 1 (n= 34) and abattoir 2 (n=
158 6). Of these 46 animals, the digested tongue was available for 43 of them. *T. gondii* DNA was detected in
159 34.8% of tissue samples (95% CI: 21.4%-50.3%) using both digested and non-digested tongue, 27.9% (95%
160 CI:15.3%-43.7%) using only digested tongue and 19.6% (95% CI: 9.4%-33.1%) using only non-digested
161 tongue. Analysis using the Chi-Square ($\chi^2= 0.59$, $p= 0.22$) and McNemar ($p= 0.34$) tests did not show a
162 statistically significant difference between methods.

163 All rodents were trapped using fattening pig food. A total of 14 rodents were captured, all of which were rats
164 (*Rattus rattus*). Five rats were females and nine males, eight were adult, five sub-adult and one juvenile. No
165 association was found between age, gender and *T. gondii* status. Overall 6 rats were positive by SAG1N-

166 PCR giving a prevalence of *T. gondii* DNA in rats as 43% (95% CI= 17%-71).

167 3.2. *T. gondii* isolation via Bioassay

168 Isolation of *T. gondii* via mouse bioassay was attempted from the 40 pigs slaughtered at the abattoirs and the
169 cat. The parasite was successfully isolated from the tongue of three pigs and the cat. Of the three isolates
170 obtained from pigs, two were from pigs PCR positive in their tissues. Agreement between PCR and isolation
171 success was slight ($\kappa= 0.15$). Overall, the parasite was isolated from 7.5% (95% CI= 1.5%-20.4%) of the
172 bioassayed pigs.

173 3.3. Genetic characterisation using MLST

174 SAG1 was the most successful probe and amplified 35, 52, and 23 additional samples than the Alt-SAG2,
175 GRA6 and SAG3 genetic markers. The SAG1 gene was successfully amplified and sequenced from 74 of 77
176 samples, of which 68 were derived from pig samples (65 from swine blood (52) and tissues (13) and 3 from
177 mouse brains), 5 from rats and one from the cat. Two infected mouse brains were obtained from tissue
178 samples taken from PCR positive pigs. DNA sequences obtained from parasites in these mouse brains were
179 identical to those obtained by direct sequencing of the tissues of the bioassayed pigs.

180 Double peaks were observed in the chromatograms after a visual inspection in 11 pig samples suggesting
181 multiple infections with different *T. gondii* strains in pigs. Double peaks were observed at one to eight
182 nucleotide sites of SAG1 (Figure 1) Alt-SAG2 or SAG3 loci. Both possible alleles were taken into account
183 in these samples for the genotype classification. Sequencing data of the 4 loci revealed that the predominant
184 allele type was atypical (46%), followed by the Type I allele (43%), the Type III allele (8%) and the Type II
185 allele (3%). Overall, the number of variant alleles was 27 (I, II-III, u-1-25), 20 (I, III, u1-18), 6 (I, III, u1-4)
186 and 11 (I, II, u1-9) for the SAG1, Alt-SAG2, SAG3 and GRA6 loci respectively (Supplementary material
187 S1). Atypical alleles were mainly associated with a Type I background in SAG1; mixed Type I-II in SAG2;
188 Type II, Type I or mixed Type I-II backgrounds in GRA6 and; Type III, Type I-II or Type I backgrounds in
189 SAG3. Table 1 shows the combination of alleles for the samples with genotyping data for 3 and 4 alleles. *T.*
190 *gondii* strains from Yucatan showed considerable diversity as allele combinations were not shared by more
191 than two samples when three and four loci were used. Overall, a total of 64 novel SNPs were noted among
192 SAG1, Alt-SAG2, GRA6 and SAG3 loci. Nine of 64 of the novel SNPs were parsimonious of which seven

193 were shared by two samples each, one was shared by three samples and one was shared by 11 samples. It is
194 noteworthy that the SNP shared by 11 animals (u-4, SAG2 allele) was non-synonymous leading to a change
195 from lysine to glutamic acid in the SAG2 locus (Supplementary material S2).

196 PCR-sequencing of additional loci was attempted with the samples for which sequencing data for the four
197 loci was achieved (Table 1). 3'SAG2, 5'SAG2, BTUB, PK1, C22-8, C29-2, L358, Apico, UPRT1 were
198 successfully sequenced for both pig53 and cat1, which were named as TgPigMx1 and TgCatMx6 based on
199 previous publications. In addition, UPRT1, UPRT7, EF1 and HP2 sequence was generated for TgCatMx6.
200 TgCatMx6 was an atypical genotype with mixed Type I, II, III and atypical alleles. TgPigMx1 was also
201 atypical but with a combination of Type I and III alleles and one atypical allele (Table 2).

202 It is usual to determine the genotypes of isolated viable *T. gondii* strains by RFLP. The RFLP patterns were,
203 therefore, predicted (Su *et al.*, 2010) and compared with the genotypes published in ToxoDB. TgCatMx6 had
204 the RFLP genotype number #154, this genotype was obtained from the isolate TgGoatUS20 from a goat in
205 the USA (Dubey *et al.*, 2011a). In contrast, the RFLP genotype of TgPigMx1 was not found. In addition, the
206 available sequenced loci GRA6, UPRT1, UPRT7, and HP2 for TgGoatUS20 were downloaded from the
207 NCBI website and compared with the SNPs of the TgCatMx6 isolate. GRA6, UPRT1, UPRT7, EF1 and HP2
208 were identical for both TgGoatUS20 and TgCatMx6, except for one SNP at the intron EF1 (Table 2).

209 3.4. Phylogenetic analysis of *T. gondii* strains from Yucatan

210 For a better understanding of the relationship between the *T. gondii* isolates from Yucatan with those from
211 North and South America, we built phylograms with the sequencing data of the 16 loci of the *T. gondii*
212 representative genotypes with North and South American origin and the genotypes TgPigMx1 and
213 TgCatMx6 obtained in this study. A total of 49 isolates obtained from animals and humans from the USA,
214 Canada, Brazil, French Guyana, Uruguay, Costa Rica and Colombia were used (Supplementary material S3).
215 Genotypes were clustered into haplogroups and thereby associated with geographical areas (Figure 2 shows
216 the NJ phylogram). Clusters A, B, C and D were composed almost exclusively of South American isolates
217 (haplogroups, 4, 5, 8, 9, 10 and 15). Clusters E, G and F were composed of isolates from North and South
218 America origin with genotypes related to Type I, III and mixed Type I and III respectively. Cluster I
219 comprised isolates exclusively from North America which had type 12 and II genotypes (ARI, B73, B41,
220 ME49, RAY). Cluster H comprised the atypical isolates COUG and GUY-2004-JAG1 with mixed Type I, II,

221 III and u-1 alleles. TgCatMx6 occupied an intermediate position between H and I clusters. The bootstrap
222 value (78) of the branch which includes both clusters I and H together with TgCatMx6 supported the close
223 relationship between these genotypes. However, the bootstrap value of the node in which TgCatMx6 was
224 grouped within the cluster I was moderate (45) indicating some divergence. TgPigMx1 was clustering
225 between cluster E and F which are found in both North and South America. The bootstrap value obtained for
226 the node which includes cluster E, F and TgPigMx1 was high (82) indicating a strong relationship but the
227 bootstrap value of the node in which TgPigMx1 was grouped within was low (18) suggesting also some
228 divergence between the isolated clustered in node E. NJ and UPGMA phylogenetic trees showed consistently
229 comparable topology supporting a robust clustering.

230 **4. Discussion**

231 Results from this study have shown a higher genetic diversity of *T. gondii* in Yucatan than in other areas of
232 Mexico as the genotypes found in this study were not shared by more than two samples and clonal types
233 were rare. Of the 33 genotypes successfully sequenced with three or more loci, only two were shared by two
234 samples, the remaining genotypes were unique. Only one genotype had Type I alleles at all three loci
235 sequenced SAG1, SAG2 and SAG3 and the remaining genotypes were observed to be mixed types I/u-(n),
236 I/III/u-(n), I/III, I/II/u-(n), I/II/III/u-(n) and I/II alleles. A total of seven RFLP genotypes have been obtained
237 in previous studies in Mexico (Dubey *et al.*, 2009, Alvarado-Esquivel *et al.*, 2011, Rico-Torres *et al.*, 2012,
238 Dubey *et al.*, 2013) from a total of 16 isolates. The clonal Type III genotype seemed more common in the
239 other studies in Mexico and was present in 4 isolates (Dubey *et al.*, 2009). However, in these studies, the
240 genetic diversity could be underestimated as RFLP has lower power in resolving identities than MLST.
241 Genetic diversity of the *T. gondii* strains from Mexico was higher than in isolates from the USA, where
242 clonal types were predominant and unique genotypes were less frequently found. For example, Velmurugan
243 *et al.*, (2009) found only 9 RFLP genotypes from 182 *T. gondii* isolates from pigs. The most common
244 genotypes were clonal Type II, a variant of clonal Type II and clonal Type III which represented 81% of the
245 isolates. Genotyping studies in Europe have found even lower genetic diversity, Djokic *et al.*, (2016)
246 recovered 41 isolates from pigs from abattoirs in France and all of them were clonal Type II by using RFLP
247 with 12 loci. In contrast, studies in Brazil found higher diversity, for example, Dubey and Su (2009) noted 58
248 different genotypes of 149 isolates from chicken and 29 (50%) of these genotypes had a single isolate each.

249 Only one isolate was of clonal Type I and five isolates were of clonal Type III, the remaining isolates had
250 recombinant or atypical genotypes, mainly with Type I and III alleles. This suggests that *T. gondii* isolates
251 from Mexico are more in line with the genetic diversity of the isolates found in Central and South America
252 than in North America and other continents. Shwab *et al.*, (2013) looked at the geographical distribution of
253 *T. gondii* genotypes by analysing 1457 *T. gondii* isolates across the continents and found 156 different
254 genotypes from 646 South/Central American isolates (24%) but only 9 genotypes from 64 European isolates
255 (14%), 10 from 102 Asian isolates (10%), 13 from 141 African isolates (9%) and 40 from 501 North
256 American isolates (8%).

257 Similarly to this study, the genetic population structure of *T. gondii* in Central America and Colombia seems
258 to lack a clear predominant genotype. For example, genotyping of 32 isolates from chickens in Costa Rica
259 using RFLP at the loci SAG1, SAG2, SAG3, BTUB and GRA6 revealed five genotypes. Five isolates had
260 Type I alleles and one isolate had Type III alleles at all loci. The remaining 26 isolates contained a
261 combination of Type I and II or I and III alleles and were divided into three genotypes (Dubey *et al.*, 2006a).
262 Genotyping of 48 isolates from chickens in Nicaragua, also using RFLP at the loci SAG1, SAG2, SAG3,
263 BTUB and GRA6, revealed eight genotypes. Six isolates had Type I alleles, three isolates had Type II alleles
264 and six isolates had Type III alleles at all loci. The remaining 29 isolates contained the combination of Type
265 I and III alleles and were divided into five genotypes (Dubey *et al.*, 2006c). In contrast, Brazil has a
266 particular genetic population structure characterised by the expansion of a few local types named as BrI-IV
267 which are not as frequently found in other regions of the continent. Chile, Fernando de Noronha (Brazil's
268 island) and West Indies have shown a different genetic population structure to the rest of South America,
269 characterised by an unusual higher frequency of Type II genotypes and less genetic diversity (Rajendran *et*
270 *al.*, 2012, Hamilton *et al.*, 2017). Theories have suggested that the Type II lineage probably originated in
271 Europe, was brought to South America and eventually expanded to become dominant in these countries.

272 Of the 64 novel SNPs found in the present study, seven were shared by two samples each (SAG1, GRA6 and
273 SAG2 loci), one was shared by three samples (SAG2 locus), one was shared by 11 samples (SAG2) and the
274 remaining SNPs were each found in a single sample. The frequency of novel SNPs suggested that these
275 genotypes were divergent from the classic Type I, II and III lineages. Due to the importance of SAG and
276 GRA genes in parasite survival, these are considered conserved sequences which may be subject to selective
277 pressure (Manger *et al.*, 1998). One SNP named as u-4 in the present study was a non-synonymous mutation

278 at the SAG2 locus which leads to a change in an amino acid and could be indicative of positive selection
279 (Bontell *et al.*, 2009). It is interesting that this mutation is shared by 11 animals suggesting it could be a
280 successful allele which may be frequent in Yucatan but more studies are needed to investigate this finding.

281 The presence of more than one allele for a given locus is characteristic of a mixed infection with two
282 different *T. gondii* strains (Ajzenberg *et al.*, 2002). Infections with multiple strains have also been reported in
283 sheep (Ajzenberg *et al.*, 2002), humans (Aspinall *et al.*, 2003), pork, lamb and beef (Aspinall *et al.*, 2002),
284 chickens (Lindström *et al.*, 2008), mice (Bajnok *et al.*, 2015), cats (Dubey *et al.*, 2009) and marsupials (Pan
285 *et al.*, 2012). Infections with multiple strains have been reported mostly in tropical areas which present
286 higher diversity of *T. gondii* genotypes (Lindström *et al.*, 2008, Dubey *et al.*, 2006b, Dubey *et al.*, 2009, Pan
287 *et al.*, 2012) such as in Mexico (Dubey *et al.*, 2009).

288 TgPigMx1 and TgCatMx6 possessed a mixture of genotypes found in both North and South America. This
289 could suggest that these genotypes were a result of genetic crosses among strains creating gene flow between
290 these geographical areas. In the present study, this admixture could have been enhanced by geographical
291 proximity as Mexico borders between these two geographical areas creating diffused boundaries between the
292 predominant genotypes from the USA and South/North America. TgCatMx6 was clustered in between
293 atypical genotypes obtained from wildlife (COUG, GUY-2004- JAG1, B41) and Type II genotypes found
294 mostly in anthropised areas. This intermediate position could suggest that these genotypes were the result of
295 hybridization between wild and anthropised strains. A spatial partitioning of *T. gondii* genotypes across
296 domestic and wild habitats has been noted with a decrease of the parasite diversity towards an area of human
297 settlement (Jian *et al.*, 2018) and the existence of wild-domestic hybrids has been noted in French Guiana,
298 Canada, and USA (Dubey *et al.*, 2011a, Dubey *et al.*, 2011b, Mercier *et al.*, 2011, Khan *et al.*, 2014). This
299 genetic exchange is likely to happen in countries where large territories are still non-anthropised and
300 therefore a co-existence between anthropised and wild ecosystems can occur. Recombination or genetic
301 exchange between strains can only occur during the sexual cycle. Thus, this genetic exchange will occur in
302 nature when a felid ingests multiple *T. gondii* strains either as a result of a single event (example, a prey with
303 multiple infections) or multiple events within a short time span (example, more than one prey harbouring one
304 or more strains each). Although the current genotyping study was not intensive enough to reveal the direct
305 source of infection by tracking genotypes, the presence of genetic exchange in this geographical area is
306 supported by the existence of multiple infections. The presence of multiple *T. gondii* strains in an

307 intermediate host gives an excellent opportunity for genetic exchange if the host is consumed by a feline
308 predator. The result of this genetic exchange could eventually lead to the creation of novel recombinant
309 strains. The discovery of a novel recombinant Type I and III in the present study in one pig TgPigMx1
310 supports this theory of sexual recombination. New recombinant genotypes have also been reported in Mexico
311 (Dubey *et al.*, 2009, Dubey *et al.*, 2013), USA (Dubey *et al.*, 2011a, Velmurugan *et al.*, 2009, Dubey *et al.*,
312 2011b) and South America (Rajendran *et al.*, 2012). This study is the first report on *T. gondii* strains in
313 Southern areas of Mexico but further research is needed for a much clearer classification of the genotypes
314 found in this geographical area in relation to the adjacent north and south parts of the continent.

315 SAG1 NPCR was used in this study to investigate the frequency of *T. gondii* DNA in pig blood and tissue
316 samples and showed high levels of infection. The 34.8% of PCR prevalence in pig tongues is consistent with
317 data obtained in a previous study in Yucatan (Hernandez-Cortazar *et al.*, 2016) and in Northern areas of
318 Mexico (Alvarado-Esquivel *et al.*, 2012, Alvarado Esquivel *et al.*, 2015). A combination of both, digested
319 and non-digested methods, produced higher levels of detection of *T. gondii* DNA (38.2%) than by using only
320 digested (32.3%) or non-digested (17.6%) samples. In a similar study in Brazil, a higher PCR prevalence was
321 also obtained by using both methods (47.1%) than using only digested (24.2%) or non-digested (36.4%)
322 (Oliveira *et al.*, 2004).

323 *T. gondii* was isolated from 7.5% of the 40 bioassayed pigs suggesting that pork consumption could be a risk
324 of *T. gondii* transmission in the locality of Yucatan. In Galván-Ramírez *et al.*, (2010), bioassay was carried
325 out in the 48 cuts of pork but slightly lower levels of isolation success (2.1%) were obtained. Nevertheless,
326 isolation studies that assessed meats from stores have obtained, in general, lower success rates of *T. gondii*
327 isolation than studies which used meat from abattoirs, where maybe the meat was fresher (Hill *et al.*, 2004).

328 Data from the present study suggested that rodents could be involved in the cycle of transmission of *T.*
329 *gondii* in pigs and suggest that rodent controls should be implemented. Several studies have demonstrated
330 that rodents can play an important role as a reservoir of *T. gondii* in pig farms (Lubroth *et al.* 1983, Weigel *et*
331 *al.*, 1995) and *T. gondii* prevalence has been seen to decrease dramatically in farms when rodent control was
332 applied (Kijlstra *et al.*, 2008).

333 **Conflict and interest**

334 The authors declare no conflicts or interests in this paper submitted.

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