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

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Abstract:

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

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

1. Introduction

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

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

A few studies have investigated the genetic diversity of the parasite in Mexico but have been mainly focussed on the northern part of the country. In patients with congenital toxoplasmosis in Mexico state, clinical samples from four mother-child pairs were genotyped by Restriction Fragment Length Polymorphism (RFLP) using the four loci SAG2, SAG3, GRA6, BTUB and only type I genotypes with unique alleles were found (Rico-Torres *et al*., 2012). Dubey *et al*., 2009 obtained 5 genotypes of thirteen *T. gondii* isolates from dogs, cats and chickens in Durango (Dubey *et al*., 2009). Four of these isolates were clonal Type III and 9 had genotypes with mixed alleles. A total of two 2 isolates were recovered from wild animals in Durango, one from a puma and one form a pigeon (Dubey *et al*., 2013, Alvarado-Esquivel *et al*., 2011). The isolate obtained from the pigeon had a genotype reported before in (Dubey *et al*., 2009) obtained from a cat in Durango. The isolate obtained from the puma had a novel genotype with mixed I, II and u-1 alleles. Rico-Torres *et al*., (2015), identified another *T. gondii* genotype obtained from a cat in Colima also with mixed I, II and III alleles. Studies in Durango and Colima used RFLP with a panel of 12 loci (Su *et al.*, 2010) as a genotyping technique.

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

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

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

2. Materials and methods

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

2.1. Origin of the samples

Samples were collected from 2013 to 2015 during summer seasons (June-September). Five hundred and eighty-six porcine blood samples were collected from pigs raised in 12 intensive farms. In addition to this cross-sectional study, 40 pig tongues and blood samples were collected from market-age pigs slaughtered in two abattoirs. Pigs slaughtered at the abattoirs were destined for human consumption. Blood and tongue samples were also collected from six 16-17 week old pigs euthanised in one of the farms (farm A) due to poor growth and respiratory problems. One cat from the same farm was also culled by the farm veterinarian as part of a measure to control the high population of cats and its brain and heart were collected for bioassay. In addition to the pig sampling, in June of 2015, forty Sherman traps (HB Sherman Traps Inc., Florida, USA) were placed during one week on one farm (farm B) infested with rodents. Traps were placed in the warehouse, the worker's break room and across all pen areas (maternity, farrowing, weaning and fattening areas). A mixture of oats with vanilla (Panti-May *et al*., 2012) was first used but this bait was replaced with pig food after observing rodent preference. Captured animals were transported to the Zoology laboratory of the Faculty of Veterinary at the Autonomous University of Yucatan where they were euthanised with pentobarbital (Pisabental®). The age of the rodents was calculated based on weight (Sridhara and Krishnamurthy, 1992). Brain, leg muscle tissue and heart tissues were collected from all trapped rodents.

2. 2. Viability of T. gondii

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

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

Porcine blood was screened by SAG1 N-PCR to assess the level of acute infection among farmed pigs. To increase the sensitivity of *T. gondii* detection in blood, DNA was extracted from the leukocyte layer (Brenier-Pinchart *et al*., 2015). To isolate the leukocyte fraction, uncoagulated blood was centrifuged at 1300g for 30 minutes. The fine white layer, corresponding to the leukocytes, was removed carefully and placed in sterile 2 ml microcentrifuge tubes. The erythrocytes remaining in the leukocyte fraction were lysed according to Gallardo and Pelayo (2013). The final pellet of leukocytes was washed twice and resuspended in 200 µl of phosphate buffered saline (PBS) for DNA extraction. Tissue DNA was extracted by dissecting 30-50 mg of the specific organ and in the case of porcine tongues, DNA was also extracted from the pellet of the digested homogenate using the same weighing portion.

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

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

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

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

The amplification and reaction conditions were performed as described elsewhere (Su *et al*., 2010, Su *et al*., 2012) with modifications to increase the sensitivity. The optimised external amplification was performed in a volume of 25 µl with 1.25 units of Hot Start Plus Taq Polymerase (HSPT) (Qiagen), 2 µl of DNA, 2 mM of $MgCl_2$, 200 µM of each dNTP and 0.35 µM of each external primer. The nested and semi-nested reaction 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 round, 2 mM of $MgCl_2$, 200 µM of each dNTP and 0.2 µM of each internal primer and conditions as in Su *et al.,* 2010. This amplification protocol was used as this had the highest sensitivity in our internal calibration reaching the detection limit of ~5.7-7.1 and 14.3 parasites per reaction in a high density of host DNA (100 ng of MDBK cells free from *T. gondii* DNA) for SAG1 and SAG2 markers. PCR-water (Qiagen) was used as a negative control in both rounds of the N-PCR and 100 ng/µl of MDBK cells spiked with ~10-100pg of *T. gondii* RH strain DNA was used as a positive control. To avoid cross-contamination, reagents and DNA were stored in small aliquots and filter tips were used in every step. PCR products were manipulated in a separate room from the PCR set up room. PCR amplifications were visualised with GelRed™ (Biotium) staining on a 1% TBE (Tris-borate-EDTA) gel with 1% to 2% of agarose (Bioline) depending on the fragment size to resolve and processed for sequencing as described before.

2.4. Data analysis

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

3. Results

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

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

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

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

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

3.2. T.gondii isolation via Bioassay

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

3.3. Genetic characterisation using MLST

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

Double peaks were observed in the chromatograms after a visual inspection in 11 pig samples suggesting multiple infections with different *T. gondii* strains in pigs. Double peaks were observed at one to eight nucleotide sites of SAG1 (Figure 1) Alt-SAG2 or SAG3 loci. Both possible alleles were taken into account in these samples for the genotype classification. Sequencing data of the 4 loci revealed that the predominant allele type was atypical (46%), followed by the Type I allele (43%), the Type III allele (8%) and the Type II 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) and 11 (I, II, u1-9) for the SAG1, Alt-SAG2, SAG3 and GRA6 loci respectively (Supplementary material S1). Atypical alleles were mainly associated with a Type I background in SAG1; mixed Type I-II in SAG2; Type II, Type I or mixed Type I-II backgrounds in GRA6 and; Type III, Type I-II or Type I backgrounds in SAG3. Table 1 shows the combination of alleles for the samples with genotyping data for 3 and 4 alleles. *T. gondii* strains from Yucatan showed considerable diversity as allele combinations were not shared by more than two samples when three and four loci were used. Overall, a total of 64 novel SNPs were noted among SAG1, Alt-SAG2, GRA6 and SAG3 loci. Nine of 64 of the novel SNPs were parsimonious of which seven were shared by two samples each, one was shared by three samples and one was shared by 11 samples. It is noteworthy that the SNP shared by 11 animals (u-4, SAG2 allele) was non-synonymous leading to a change from lysine to glutamic acid in the SAG2 locus (Supplementary material S2).

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

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

3.4. Phylogenetic analysis of T. gondii strains from Yucatan

For a better understanding of the relationship between the *T. gondii* isolates from Yucatan with those from North and South America, we built phylograms with the sequencing data of the 16 loci of the *T. gondii* representative genotypes with North and South American origin and the genotypes TgPigMx1 and TgCatMx6 obtained in this study. A total of 49 isolates obtained from animals and humans from the USA, Canada, Brazil, French Guyana, Uruguay, Costa Rica and Colombia were used (Supplementary material S3). Genotypes were clustered into haplogroups and thereby associated with geographical areas (Figure 2 shows the NJ phylogram). Clusters A, B, C and D were composed almost exclusively of South American isolates (haplogroups, 4, 5, 8, 9, 10 and 15). Clusters E, G and F were composed of isolates from North and South America origin with genotypes related to Type I, III and mixed Type I and III respectively. Cluster I comprised isolates exclusively from North America which had type 12 and II genotypes (ARI, B73, B41, ME49, RAY). Cluster H comprised the atypical isolates COUG and GUY-2004-JAG1 with mixed Type I, II, III and u-1 alleles. TgCatMx6 occupied an intermediate position between H and I clusters. The bootstrap value (78) of the branch which includes both clusters I and H together with TgCatMx6 supported the close relationship between these genotypes. However, the bootstrap value of the node in which TgCatMx6 was grouped within the cluster I was moderate (45) indicating some divergence. TgPigMx1 was clustering between cluster E and F which are found in both North and South America. The bootstrap value obtained for the node which includes cluster E, F and TgPigMx1 was high (82) indicating a strong relationship but the bootstrap value of the node in which TgPigMx1 was grouped within was low (18) suggesting also some divergence between the isolated clustered in node E. NJ and UPGMA phylogenetic trees showed consistently comparable topology supporting a robust clustering.

4. Discussion

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

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

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

Of the 64 novel SNPs found in the present study, seven were shared by two samples each (SAG1, GRA6 and SAG2 loci), one was shared by three samples (SAG2 locus), one was shared by 11 samples (SAG2) and the remaining SNPs were each found in a single sample. The frequency of novel SNPs suggested that these genotypes were divergent from the classic Type I, II and III lineages. Due to the importance of SAG and GRA genes in parasite survival, these are considered conserved sequences which may be subject to selective pressure (Manger *et al*., 1998). One SNP named as u-4 in the present study was a non-synonymous mutation at the SAG2 locus which leads to a change in an amino acid and could be indicative of positive selection (Bontell *et al*., 2009). It is interesting that this mutation is shared by 11 animals suggesting it could be a successful allele which may be frequent in Yucatan but more studies are needed to investigate this finding.

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

TgPigMx1 and TgCatMx6 possessed a mixture of genotypes found in both North and South America. This could suggest that these genotypes were a result of genetic crosses among strains creating gene flow between these geographical areas. In the present study, this admixture could have been enhanced by geographical proximity as Mexico borders between these two geographical areas creating diffused boundaries between the predominant genotypes from the USA and South/North America. TgCatMx6 was clustered in between atypical genotypes obtained from wildlife (COUG, GUY-2004- JAG1, B41) and Type II genotypes found mostly in anthropised areas. This intermediate position could suggest that these genotypes were the result of hybridization between wild and anthropised strains. A spatial partitioning of *T. gondii* genotypes across domestic and wild habitats has been noted with a decrease of the parasite diversity towards an area of human settlement (Jian *et al*., 2018) and the existence of wild-domestic hybrids has been noted in French Guiana, Canada, and USA (Dubey *et al*., 2011a, Dubey *et al*., 2011b, Mercier *et al*., 2011, Khan *et al*., 2014). This genetic exchange is likely to happen in countries where large territories are still non-anthropised and therefore a co-existence between anthropised and wild ecosystems can occur. Recombination or genetic exchange between strains can only occur during the sexual cycle. Thus, this genetic exchange will occur in nature when a felid ingests multiple *T. gondii* strains either as a result of a single event (example, a prey with multiple infections) or multiple events within a short time span (example, more than one prey harbouring one or more strains each). Although the current genotyping study was not intensive enough to reveal the direct source of infection by tracking genotypes, the presence of genetic exchange in this geographical area is supported by the existence of multiple infections. The presence of multiple *T. gondii* strains in an intermediate host gives an excellent opportunity for genetic exchange if the host is consumed by a feline predator. The result of this genetic exchange could eventually lead to the creation of novel recombinant strains. The discovery of a novel recombinant Type I and III in the present study in one pig TgPigMx1 supports this theory of sexual recombination. New recombinant genotypes have also been reported in Mexico (Dubey *et al*., 2009, Dubey *et al*., 2013), USA (Dubey *et al*., 2011a, Velmurugan *et al*., 2009, Dubey *et al*., 2011b) and South America (Rajendran *et al*., 2012). This study is the first report on *T. gondii* strains in Southern areas of Mexico but further research is needed for a much clearer classification of the genotypes found in this geographical area in relation to the adjacent north and south parts of the continent.

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

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

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

Conflict and interest

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

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