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Toxoplasma gondii in women with recent abortion from Southern Mexico

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ABSTRACT

Objective: To estimate the presence, parasite burden, percentage of avidity and risk factors to seropositivity for *Toxoplasma gondii* (*T. gondii*) in women with recent abortion attended at the two main hospitals from Merida, Yucatan, Mexico.

Methods: Seroprevalence and IgG avidity were estimated from 161 blood samples from aborting women and risk factors associated with serological status were determined. Only 130 from 161 samples were analyzed by quantitative PCR (qPCR) and a nested-PCR (nPCR) to detect *T. gondii*-DNA.

Results: Seroprevalence of *Toxoplasma* IgM and IgG was 3.72% (6/161) and 59.00% (95/161), respectively; 98.94% (94/95) of the women showed high-avidity index and only 1.05% (1/95) demonstrated low-avidity. For qPCR, 3.84% (5/130) of the samples were detected with *T. gondii*-DNA and all positive samples showed a low parasite load (< 1 parasite/mL). For nPCR, 6.92% (9/130) of positivity was found. Logistic regression analysis showed a positive association between IgG-seropositivity and potable-drinking-water intake in households ($P < 0.020$, odds ratio = 11.62, 95% confidence interval = 0.011–0.683).

Conclusions: In this study, the detection of *T. gondii*-DNA in IgG-positive women suggests in some cases active parasitemia, even when they demonstrated high-avidity index. Reinfection cases could be presented due to the high circulation of the parasite in the study region. *T. gondii* could play an important role as cause of abortion and municipal potable-water could be an important risk factor of infection.

1. Introduction

Acute infection of *Toxoplasma gondii* (*T. gondii*) during pregnancy is detrimental for the developing fetus[1]. Toxoplasmosis is considered a public health problem and a neglected disease[2]. Congenital toxoplasmosis has been

associated with abortion and stillbirth, as well as neonatal mortality and morbidity[3], especially if it occurs in the first trimester of pregnancy[4]. Populations at risk for congenital toxoplasmosis include immunocompetent women when become newly infected during pregnancy or challenged with atypical parasite strains as well immunosuppressed mothers with HIV/AIDS[5]. The infection is transmitted either by the ingestion of oocysts from water and soil, or the tissue cysts from raw or undercooked infected meat with *T. gondii*[6]. Many factors may impact the epidemiology of *T. gondii* such as environmental conditions (e.g. climate), local density of felines, management of livestock and habits of meat consumption[7]. In the United States, approximately 1 in 10 000 normal births are affected by congenital toxoplasmosis[1]. In Mexico, the frequency of congenital infection by *T. gondii* is estimated in 2 cases per

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The study protocol was performed according to the Helsinki declaration and approved by Bioethic Committee from the Regional Research Center "Dr. Hideyo Noguchi". Informed written consent was obtained from each participant before sampling.

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1 000 newborns[8]. Furthermore, it has been reported that in Mexico 6.1% to 34.9% of pregnant women have been exposed to *T. gondii*[9]. A *T. gondii* seroprevalence of 6.7% to 55% has been reported in women with abortion in Mexico[9-11]. In the state of Yucatan, Mexico, the presence of the parasite DNA has been reported in 19% of women with clinical records of recent abortion[11].

More recently, studies of *T. gondii* seropositivity in women with history of miscarriage and stillbirths were associated with consumption of raw or undercooked meat[10,12]. There is little information about the epidemiology of *T. gondii* infection in women suffering from recent abortions, particularly when looking at potential sources of infection that could be influencing the high seroprevalence reported in the southern region of Mexico, where no program for early diagnosis exists. The aim of the present study was to estimate the presence, burden parasite, percentage of avidity and risk factors to seropositivity for *T. gondii* in women with recent abortion attended at two hospitals in the city of Merida, Yucatan, Mexico.

2. Materials and methods

2.1. Study area and population

A cross sectional study was performed from 161 blood samples obtained from women attended for abortion and prenatal care at the General Hospital "Dr. Agustín O'Horan" and the General Hospital No. 12 "Benito Juárez", both located in the city of Merida, Yucatan, Mexico (latitude 20°57'57" and longitude 89°37'23"), from September 2013 to June 2014. Patients were explained the purpose and procedures of the study and signed a letter of informed consent to be included in the study. Inclusion criteria were patients with recent abortion residing in the state of Yucatan, Mexico. Exclusion criteria of patients with recent abortion were those not living in the study area, with a history of induced abortion or endometrial abnormalities. The elimination criteria were those patients not responding the questionnaire or some of the biological samples were not obtained. The study protocol was performed according to the Helsinki declaration and approved by Bioethic Committee from the Regional Research Center "Dr. Hideyo Noguchi".

2.2. Sampling

Blood samples were obtained by venipuncture of the radial vein. Two blood samples were collected from each patient; 5 mL of whole blood sample was collected in tubes PAX-gene (BD-QIAGEN, num. cat. 761125) to preserve the DNA until purification and 5 mL of a second sample without anticoagulant using sterile Vacutainer® tubes type was centrifuged at 2000 r/min for 10 min to obtain the serum. Both, the purified DNA and the sera were stored at -20 °C until analysis.

2.3. Detection of IgG and IgM antibodies against *T. gondii*

For detection of IgM antibodies, the *Toxoplasma* IgM capture ELISA (Vircell, Spain, num. cat. M1027) was used. For IgG antibodies, the toxo IgG (Proscience Human-GmbH, Germany, num. cat. 51209) was used. Both tests were performed following the manufacturer's instructions. The optical density (OD) was measured in a spectrophotometer (XMarkTM, Bio-Rad) at 450 nm; all samples were analyzed by duplicate.

2.4. Serological TORCH assays

The presence of agents included in the TORCH test (*T. gondii*, rubella, cytomegalovirus and herpes simplex virus) was analyzed to determine the possible role of other causes that may be involved in the cases of abortion. The commercial kits used were: Rubella IgM Capture ELISA (Vircell, Spain, num. cat. M1026), Cytomegalovirus IgM Capture ELISA (Vircell, Spain, M1004) and Enzygnost Anti-HSV/IgM (SIEMENS, num. cat. OWNX15). Manufacturers' guidelines were followed for the serological evaluation.

2.5. Avidity test

All positive samples to *T. gondii* IgG antibodies underwent the avidity test to exclude recent infections. The technique used is described by Cozon *et al.*[13], using the same commercial kit for the detection of IgG antibodies. Sera were diluted at 1:100 and placed by duplicates on 96 well plates coated with *T. gondii* antigens. At the end of the first incubation, unbound components were removed in the washing step. A slight modification was done at this time, where one of the replicates was washed with 300 µL of urea 6 mol/L three times for 5 min each time; the other replicates were washed five times during 30 s each time with 300 µL of the solution provided by the commercial kit. Absorbance was measured at 450 nm on a spectrophotometer (XMarkTM, Bio-Rad). Results were expressed in percentage of avidity dividing the OD after washing with urea between OD after washing with the buffer ×100. A value > 35% is associated with chronic infection and could absolutely exclude a recent (< 3 months) infection.

2.6. Molecular detection of *T. gondii* and parasite load determination by quantitative PCR (qPCR)

DNA was purified from whole blood samples according to Jalal *et al.*[14]. Subsequently, the protocol of the commercial kit DNeasy Blood and Tissue (QIAGEN) was followed. The qPCR was performed in order to amplify a fragment of 62 bp B1 gene present 35 times in the genome of *T. gondii*. The primers used were forward 5'CTAGTATCGTGCGGCAATGTG3' and reverse 5'GGCAGCGTCTCTTCCTCTTTT3' and TaqMan probe 5'(6-FAM)

-CCACCTCGCCTCTTGG-(NFQ-MGB) 3'^[15]. For amplification, the TaqMan® Universal PCR Master Mix IX (PE Applied Biosystems) was used reaction with an adjusted concentration of 5 mmol/L MgCl₂, 0.90 μmol/L of forward and reverse primers, 0.25 μmol/L of FAM-labeled probe and 4 μL DNA sample in a final volume of 25 μL. The carryover contamination was prevented by using the heat-labile enzyme uracil N-glycosylase (UNG AmpErase®, PE Applied Biosystems), included in the Universal PCR Master Mix. The reaction mixture was initially incubated for 2 min at 50 °C to allow the action of the UNG. As controls, the DNA obtained from an individual without previous contact with the parasite was used. Amplification conditions were an initial denaturation at 95 °C/10 min, followed by 40 cycles at 95 °C/15 s and 60 °C/1 min in a real time thermocycler C1000 CFX 96TM (Bio-Rad). A simple fluorescence reading of each sample was taken in the alignment step-elongation. The standard curve was constructed using serial dilutions of known amounts of DNA from *T. gondii* tachyzoites (1 × 10⁵ to 1 × 10²), to perform the absolute quantification by means of PCR thermocycler software in real time CFX 96TM C1000 (Bio-Rad). Sensitivity and detection limit of the qPCR was evaluated. Results were expressed as *T. gondii* tachyzoites equivalent per milliliter of blood of the patients studied. The presence of endogenous PCR inhibitors in each DNA sample was assessed by qPCR amplification of a GAPDH gene.

2.7. Nested PCR (nPCR)

A nPCR was used to amplify a fragment of 390 bp *SAG1* gene (major surface protein of *T. gondii*), using a 96 wells Veriti thermocycler (Applied Biosystems). The first round of amplification was performed with the external primers forward (5'-GTTCTAACCACGCACCCTGAG-3') and reverse (5'-AAGAGTGGGAGGCTCTGTGA-3'); in the second round amplification, the internal primers used were: forward (5'-CAATGTGCACCTGTAGGAAGC-3') and reverse (5'-GTGGTCTCCGTCGGTGTGAG-3'). The first amplification reaction was carried out with IX PCR buffer (Promega) with an adjusted MgCl₂ concentration of 2 mmol/L, 0.8 mmol/L of dNTPs, 0.5 μmol/L for both forward and reverse primers; 1.5 IU *Taq* polymerase, and 2 μL of DNA samples in a final volume of 25 μL and the PCR conditions were 95 °C/5 min, followed by 30 cycles of 94 °C/30 s, 55 °C/1 min and 72 °C/2 min. The second PCR had the same characteristics as the first, except that in this case, the concentration used for forward and reverse primers was modified to 0.3 μmol/L and used 2 μL of PCR product from the first round and amplifications conditions were 95 °C/5min, followed by 35 cycles of 94 °C/30 s, 60 °C/1 min and 72 °C/1 min 30 seg. The positive and negative controls (DNA of *T. gondii* and reagents only, respectively) were included in each nPCR analysis. The amplification products were revealed on 1.5% agarose gel stained with ethidium bromide.

2.8. Risk factors

A questionnaire was used to obtain information regarding risk factors that could be associated with the IgG seropositivity. The demographic data included age, residence and occupation. Clinical data were number of pregnancies, childbirth, cesarean section, abortions, presence of any immune disease and clinical condition before the event of abortion (bleeding, pain and temperature). Other data included contact with cats, eating raw or undercooked meat, raw water intake, consumption of raw vegetables and gardening activities.

Serological and molecular results were classified as being in contact with the agent (-IgM, +IgG and -PCR), reinfection (-IgM, +IgG and +PCR), acute (+IgM, -IgG and -PCR or +IgM, +IgG and -PCR), active (-IgM, -IgG and +PCR) or negative (-IgM, -IgG and -PCR).

2.9. Statistical analysis

A *Chi* square test was used to study risk factors (using Fisher's exact test when values were less than 5) associated with serological presence of IgG antibodies to *T. gondii*. The software EpiData v.3.0 (CDC, Atlanta, GA) was used. Factors where the *P* value was less than 0.20, underwent a multivariate logistic regression analysis to accurately determine the association of factors with IgG seropositivity of patients. Logistic regression analysis was carried out using the SPSS software v.17.

3. Results

3.1. Serology and avidity

Overall, from the 161 samples 59.00% (95/161) and 3.72% (6/161) had IgG and IgM antibodies against *T. gondii*, respectively. Results of the serological TORCH assays were: 3.72% (6/161) for *T. gondii*, 0.00% for rubella (0/161), 0.62% (1/161) for herpes simplex virus and 0.62% (1/161) for cytomegalovirus. From the total positive samples to *T. gondii* IgG antibodies, a low avidity index was found in only one sample (1.05%) without presence of IgM antibodies; in contrast, a high avidity index was found in 98.94% (94/95) of the samples, indicating that the infection was latent and was acquired more than four months before abortion.

3.2. qPCR and nPCR for *T. gondii* detection

The presence of *T. gondii* DNA detected by qPCR was 3.84% (5/130), with a detection limit of the standard curve of 1 parasite/mL of blood. The number of parasites found in the five positive samples was less than 1 parasite/mL of blood. In the nPCR, 6.92% (9/130) of the samples were positive. An example of *SAG1* gene amplified in an agarose gel and stained with 1.5% ethidium bromide is shown in Figure 1.

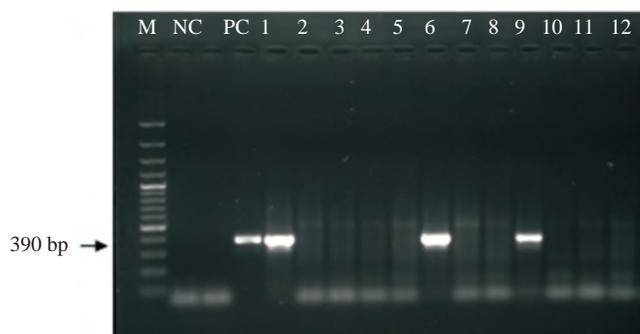


Figure 1. Electrophoresis in 1.5% agarose and ethidium bromide staining of nPCR amplified gene *SAG1* products of *T. gondii*.

M: Molecular weight marker (gene ruler 100 bp plus DNA ladder 100 to 3000 bp, num. cat. SM0321); NC: Negative control (master mix without DNA); PC: Positive control (*T. gondii* DNA culture); lanes 1, 6 and 9: Samples from *T. gondii* positive women; lanes 2, 3, 4, 5, 7, 8, 10, 11 and 12: Samples from *T. gondii* negative women.

The results of the different serological and molecular tests, and their combination are presented in Table 1; most cases were classified as being of contact (53.07%) and 33.07% were negative to all tests. Also, Table 2 shows the positive and negative cases of both qPCR and nPCR.

Table 1

Toxoplasmosis case classification based on serological and molecular tests from women with spontaneous abortion.

Type of infection	IgM	IgG	qPCR and nPCR	n (cases)	Frequency (%)
Contact	-	+	-	69	53.07
Reinfection	-	+	+	8	6.15
Acute	+	-	-	2	1.53
	+	+	-	4	3.07
Active	-	-	+	4	3.07
Negative	-	-	-	43	33.07
Total				130	100.00

Table 2

Positive and negative cases of both qPCR and nPCR from women with spontaneous abortion.

qPCR	nPCR	Cases	Frequency (%)
+	+	2	1.53
+	-	3	2.30
-	+	7	5.38
-	-	118	90.76
Total		130	100.00

3.3. Risk factors

Results of the *Chi* square test (Table 3) indicated that consumption of undercooked meat, consumption of unwashed raw vegetables, keeping cats as pets and use of potable water to prepare drinks were factors associated with seropositivity ($P \leq 0.20$). However, after the multivariate logistic regression analysis (Table 4) the only risk factor statistically significant was drinking potable water ($P < 0.020$, odds ratio = 11.62, 95% confidence interval = 0.011–0.683).

Table 3

Univariate analysis of behavioral factors of women with recent abortion and infection with *T. gondii*.

Characteristic	Category	n	n (%) positive to <i>T. gondii</i>	P value
Undercooked meat consumption	No	90	61 (96.82)	0.04*
	Yes	7	2 (3.17)	
Cooked meat consumption	No	83	51 (54.25)	0.64
	Yes	76	43 (45.74)	
Raw vegetables consumption	No	69	42 (48.83)	0.57
	Yes	80	44 (51.16)	
Washed raw vegetables consumption	No	73	37 (84.09)	0.049*
	Yes	8	7 (15.90)	
Disinfected raw vegetables consumption	No	32	18 (46.15)	0.76
	Yes	42	21 (53.84)	
Handling of raw meat	No	44	24 (25.53)	0.54
	Yes	114	70 (74.46)	
Food consumption in the streets	No	67	44 (46.80)	0.23
	Yes	91	50 (53.19)	
Consumption of fresh milk	No	135	81 (86.17)	0.75
	Yes	24	13 (13.82)	
Consumption of boiled fresh milk	No	14	9 (69.23)	0.41
	Yes	9	4 (30.76)	
Consumption of artisanal cheese	No	151	91 (96.80)	0.27*
	Yes	8	3 (3.19)	
Gardening activities	No	120	68 (72.34)	0.35
	Yes	39	26 (27.65)	
Use gloves when gardening activities	No	30	21 (80.76)	0.33*
	Yes	9	5 (19.23)	
Presence of cats at home	No	81	46 (49.46)	0.63
	Yes	76	47 (50.53)	
Cats as pets	No	127	80 (85.10)	0.07
	Yes	32	14 (14.89)	
Use of potable water at home	No	90	59 (93.65)	0.47*
	Yes	7	4 (6.34)	
Drinking water (potable vs. purified)	No	75	42 (66.66)	0.0003
	Yes	22	21 (33.33)	
Using water to prepare drinks (well vs. purified + potable)	No	75	42 (65.62)	0.0003
	Yes	23	22 (34.37)	

*: Fisher exact test.

Table 4

Multivariate logistic regression analysis of behavioral factors in women with abortion.

Characteristic	P value	OR	CI (95%)
Consumption of undercooked meat	0.055	5.259	0.962–28.739
Consumption of raw vegetables not washed	0.089	0.155	0.018–1.330
Cats as pets	0.051	2.188	0.997–4.802
Drinking potable water	0.020	11.620	0.011–0.683

OR: Odds ratio; CI: Confidence interval.

4. Discussion

Results obtained in this study indicate that a high percentage of women who suffer abortions have had previous contact with the parasite. These results are similar to that reported in 2013, where 55% of women with spontaneous abortion have IgG antibodies against *T. gondii*[11]. In contrast, in Northern Mexico (Durango City), it reported a seropositivity of 6.7% and 0.6% for IgG and IgM respectively, in women with spontaneous abortions[10]. The noticeable difference between seroprevalence reported among these regions may be due to the climatic conditions, particularly in the tropical-humid regions[16]. The results described here show that

37.26% of women with abortions had no previous contact with *T. gondii* at the time of conception, meaning that a high percentage of pregnant women are at risk of exposure to *T. gondii* and consequent primary infection during pregnancy.

The IgG avidity assay is a good reference for excluding a recently acquired toxoplasmosis infection in an IgM-positive serum sample[17,18]; *T. gondii* IgM antibodies may remain detectable for months or even years after the initial infection and thus the avidity test allows a better diagnosis to take resolutions during the pregnancy[19]. In the present study, four women with presence of IgM antibodies and high avidity index were found, thus discarding a recent infection for at least the last three months; besides, only 1.05% (1/95) of studied cases showed a low avidity index without presence of IgM antibodies. With these results, the interpretation may be difficult and it is advisable to follow the patient sera to confirm a recent infection[17]. For the cases with presence of IgM and absence of IgG antibodies, another paired test is recommended, considering that IgG synthesis is 1–3 weeks after the surge of IgM.

The presence of *T. gondii* DNA in eight cases found in the present study (6.15%) showed a high avidity index classifying them as a reinfection. Several cases of congenital toxoplasmosis transmitted from immunized mothers before conception have been reported[20,21].

One of those cases involving a rare atypical genotype isolated from the blood of the newborn in Europe also described in South America, concluding that the immunity acquired by certain strains probably does not protect against reinfection by atypical strains[20], and the immunity created by the ingestion of cysts contained in the meat does not protect against reinfection with oocysts due to the antigenic differences between tachyzoites and oocysts[22]. In this study, four patients with only presence of *T. gondii* DNA without the presence of IgG and IgM antibodies were found. Considering the total of positive cases with presence of *T. gondii* DNA in both qPCR and nPCR assays, it suggests that these patients had a recent infection and *T. gondii* might be the probable cause of the abortions. It has also been showed that a positive serological result is only indicative of previous infection, whereas direct detection of *T. gondii* in blood or other tissue sample categorically confirms the parasite presence leading to the diagnosis of primary, reactivated or chronic toxoplasmosis[23].

It is important to mention, that there are repetitive DNA sequences that are often used for the detection of *T. gondii* in biological samples, including the 35-copy B1 gene and the 300-copy 529 bp repeat element gene sequences[24]. The 529 bp repeat element has been reported to be more sensitive than the B1 gene[25]. However, the use of TaqMan probe in real-time PCR for B1 gene is a way to reach to a specific result, demonstrating a high sensitivity[26]. In the present study, results of the different PCR assays indicate that nPCR is more sensitive than qPCR, coinciding with previous reports[27]. However, qPCR makes the quantification of parasites and could still be used for the diagnosis of toxoplasmosis as well as in their pathogenesis and treatment-response studies.

The information obtained from the questionnaire in PCR positive

women indicate several characteristics that might be influencing the results such as activities of gardening without gloves, presence of cats in the house, having cats as pets, unwashed vegetable consumption, eating undercooked meat and drinking water for beverages. Only drinking potable water was statistically significant. Several reports of outbreaks of waterborne toxoplasmosis in different parts of the world have been described[28]. A large number of individuals were affected with oocysts, found in the soil and water contaminated with cat feces[6]. Recently, an ELISA against TgERP (sporozoite-specific embryogenesis related protein) was used in human samples, with the aim of differentiating oocyst-acquired infections from those acquired through tissue cysts; they showed the possibility of a continuous contamination of drinking water with *T. gondii* oocysts in this endemic setting[29]. It must be emphasized that a single oocyst can cause infection[30]. It has already been demonstrated that the oocysts are resistant to most methods of microbial inactivation during water-treatment[31].

Therefore, it is necessary to investigate the presence of oocysts in different water sources that supply the studied areas in Yucatan, Mexico, and other potential sources of infection such as consumption of pork, in order to determine the role of these two sources of infection in general population. The implementing of strategies like hygienic educational programs to prevent about risk for toxoplasmosis reduces fatal results in products for pregnant women.

The seroprevalence here found suggest that *T. gondii* is widely circulating and allowing the infection and/or reinfection of pregnant women. The detection of parasite DNA suggests a recent infection or a reinfection, where *T. gondii* could be playing an important role in the abortions. The low parasite load found in women with abortion suggests that the implementation of molecular techniques such as qPCR and nPCR are required to perform an accurate diagnosis allowing to provide an oportune treatment.

Conflict of interest statement

We declare that we have no conflict of interest.

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