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Original Article

Serological Evaluation and Molecular Genotyping of *Toxoplasma gondii* in Pregnant Women in Meshkin-Shahr District, Northwestern Iran

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Abstract

Background: We aimed to investigate the serological and molecular characteristics of *Toxoplasma gondii* infection among pregnant women and their aborted placentas in Meshkin-Shahr City during 2019-2020.

Methods: Blood samples of 210 pregnant women were evaluated for anti-*T. gondii* antibodies and related risk factors were determined. Also, the sera of aborted women and their buffy coats and aborted placenta tissues were used to detect anti-*Toxoplasma* antibodies and the parasite's DNA, respectively. The parasite genotypes were determined by the PCR-RFLP using the *SAG3* gene.

Results: The overall prevalence of anti-*Toxoplasma* IgG was 24.3% and only 1% of participants were seropositive for the IgM antibody. There was a significant relationship between raw or unwashed vegetable consumption, contact with soil, vegetable/fruit washing type, and seropositivity ($P<0.05$). During pregnancy, 4.7% of women encountered an abortion and 30% and 50% of cases were positive for IgG antibodies before and after abortion, respectively. Only two cases were IgM seropositive after abortion. In the avidity IgG test, 20% of cases showed low avidity. BLAST and phylogenetic analysis exhibited that all isolates belonged to the type III *T. gondii* genotype. Although two women with spontaneous abortions showed seropositivity for IgM *T. gondii* antibody, parasite DNA was detected in three cases.

Conclusion: The seroprevalence of *Toxoplasma* infection is not high in pregnant women. Seropositive women are not safe from congenital transmission. *T. gondii* type III is the etiology of fetus infection in mothers with spontaneous abortion. It seems that screening and essential care are still necessary during pregnancy.

Introduction

Toxoplasma gondii is a cosmopolitan intracellular protist that is found among one-third of the human population (1). According to the recent classification by the Food and Agriculture Organization of the United Nations and the WHO, toxoplasmosis is fourth rank among 24 harmful foodborne pathogens (2).

T. gondii is usually transmitted to humans by eating raw or undercooked meat containing tissue cysts (3), food contaminated by cat oocysts (4), and blood transfusion, organ transplantation, and congenitally (3).

Although the infection has no clinical symptoms in immunocompetent individuals (5), the clinical aspects are serious in subjects with immature or impaired immune systems, and seronegative pregnant women and their newborns (6, 7). The risks of transmitting the infection to the fetus in the first, second, and third trimesters of pregnancy were different and are estimated to be about 15%, 44%, and 71%, respectively (8). The first and second trimesters of pregnancy are very important in terms of contamination of the fetus, and the severity of the clinical symptoms is more life-threatening (9). After the congenital transmission of the parasite through the placenta, the *T. gondii* rapidly enters immune-privileged organs such as the liver, eye, and brain (10), which may lead to severe complications (11-13).

The global prevalence of *T. gondii* infection in women with abortion is 10% to 33% (14-16). Additionally, the overall seroprevalence of *T. gondii* infection as a prevalent parasite among childbearing-age Iranian women was 39.9% (17). Another study found a 45.1% seroprevalence of toxoplasmosis in immunocompromised patients in Iran (18).

Due to the lack of a study on pregnant women in Meshkin-Shahr City, the seroprevalence of anti-*T gondii* antibodies and the incidence of spontaneous abortion using ELISA and PCR methods have been conducted for the first time in this region.

Materials and Methods

Ethics approval and informed consent

The Ethics Committee of Ardabil University of Medical Sciences approved the study (Ethics no: IR.ARUMS.REC.1397.169). A declaration of volunteer participation and informed consent were obtained from each participant before enrolment.

Study area and sample collection

This cross-sectional survey was performed on pregnant women in Meshkin-Shahr City, northern Iran, 2019-2020. A predesigned questionnaire consisting of demographic criteria and related risk factors was filled out for each woman. Of 210 pregnant women who had no history of other diseases were included in the study. Blood samples (about 5 mL) were collected for serological evaluation. All participants were then followed up for possible miscarriage during their pregnancy. In addition, buffy coats and placenta samples of women who had abortions during pregnancy were evaluated using serological and molecular methods.

Serological assays

Anti-*T. gondii* IgM and IgG antibodies were detected using a commercial ELISA kit (PishtazTeb, Tehran, Iran) according to the manufacturer's protocol. The ELISA Avidity kit (EUROIMMUN, Germany) was used to determine the approximate time of *Toxoplasma* infection in women who had abortions and were positive for anti-*Toxoplasma* antibodies.

Molecular detection

The genomic DNA from all buffy coat samples was extracted by a DNA extraction kit (Sinaclon, Iran). The repetitive element (RE)

gene as a sensitive and specific target using the PCR method was used for the screening and early detection of T. gondii. Conventional PCR was conducted using the 529 bp RE gene in a final volume of 25 µL (19). Summarily, 12.5 µL master mix (PCR Mastermix, Cat. no. MM2062, TC Clone, Tehran, Iran), 5 µL template DNA, 1 µL of each forward and reverse primers (10 pmol/µL) [TOXF (5′CGCTGCAGGGAGGAAGACGAAAGTTG-3′) and TOXR (5′-CGCTGCAGACACAGTGCATCTGGATT-3′)], and 5.5 µL of ddH2O. Amplification was performed with the first and final denaturation including for 7 min at 95 °C and 45 s at 95 °C for 35 cycles, respectively, annealing for 45 s at 60 °C, and the first and final extension at 72 °C for 45 s and 10 min, respectively. The standard RH strain of T. gondii, as the positive control, and distilled water as the negative control were used. Finally, the PCR products were run separated on 1.5% agarose gel and stained with safe stain (Sinaclon) in Tris acetate-EDTA buffer for observation of the 529 bp bands, under a gel-documentation apparatus (UVP, Upland, CA, USA). Genotyping was carried out on the positive samples using the *SAG3* marker by nested-PCR method (20, 21). Next, the products were digested using the BcnI (NciI) restriction enzyme (Cat. No. ER0061, Thermo Fisher Scientific, USA). The restriction fragments were electrophoresed on the 1.5% agarose gel and visualized under ultraviolet illumination. For determining the *T. gondii* genotype, sequence analysis of *SAG3* positive samples (three isolates) was performed and aligned with BioEdit Multiple Alignment software (22). The obtained sequences were compared with other sequences registered in the NCBI GenBank. Phylogenetic tree was constructed using Tamura-Nei model of the maximum likelihood method by Molecular & Evolution Genetic Analysis software version 6 (MEGA 6). Bootstrap value was considered based on 1000 replications for determining the reliability of topology of the tree.

Statistical analysis

All data were analyzed using the Chi-square test or t-test by SPSS (version 24) software (IBM Corp., Armonk, NY, USA) with a *P*-value<0.05 as statistically significant. A logistic regression analysis was used to identify the associations between explanatory variables and seropositivity. All variables with a coefficient *P*-value≤0.2 in the univariable model were entered into the multivariable modeling to show the effects of confounding risk variables that would explain the occurrence of toxoplasmosis.

Results

Of 210 pregnant women, with an average age of 25.9±7.99 years, the overall seroprevalence was 24.3%. Of these, 2 (1 %) and 49 (23.3 %) samples were positive for IgM and IgG antibodies, respectively. These women had an abortion (22.4%), and stillbirth (3.8%) history. Most of the participants were rural, unemployed, and had diplomas. Results of the univariate and multivariate data analysis showed that anti-*T. gondii* IgG seropositivity is significantly associated with raw or unwashed vegetable consumption, contact with soil, and vegetable/fruit washing type (P<0.05) (Table 1). In this study, women were monitored during pregnancy, and 4.7% (10/210) encountered abortion. Their mean ages were between 28.9±9.06 years and had 2 time pregnancies. Three cases had a history of previous abortion and most of the abortions had occurred in the first trimester. Results of the ELISA test showed that 3 (30%) and 5 (50%) cases were positive for IgG antibodies before and after abortion, respectively. Only two cases were positive for the IgM antibody. These five samples were evaluated by the IgG avidity test, in which two cases showed low avidity (Table 2). Either molecular analysis targeting *RE* or

SAG3 genes showed that *Toxoplasma* DNA was detected in 30% (3/10) and 20% (2/10) of the buffy coat and placenta samples, respectively (Fig 1). PCR-RFLP results have shown that all isolates belonged to the *T. gondii* type III genotype (Fig. 2). Three isolates were sent to sequence by the *SAG3* gene and registered in the GenBank database (Accession no. MW076233). The nucleotide blast of these sequences at the NCBI (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi) revealed 100% homologies with *T. gondii* isolated that was entrusted to the GenBank (Fig. 3).

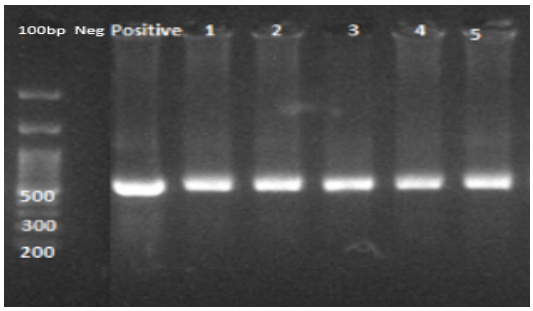

Fig. 1: Gel shows a band for the positive control (Positive), but not the negative control (Neg), Lanes 1, and 2: positive blood samples, lines 3-5 show representative placenta samples with 529 bp bands.1: Ladder, 100 bp marker

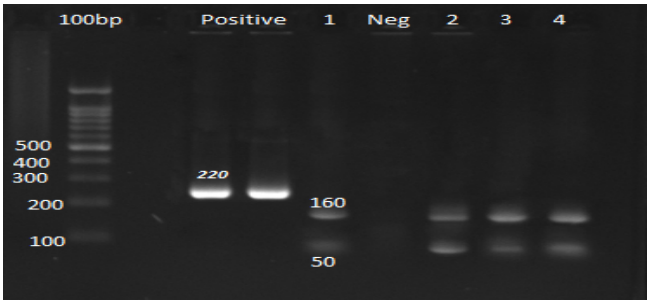

Fig. 2: Amplification of the *T. gondii SAG3* gene by PCR. The gen was then cut by the *Nci I* enzyme and showed the type III genotype in this area. Lanes 2,3 are undigested positive samples. Lane 1 digested buffy coat, lanes 1-4 digested placenta samples, Ladder, 100 bp

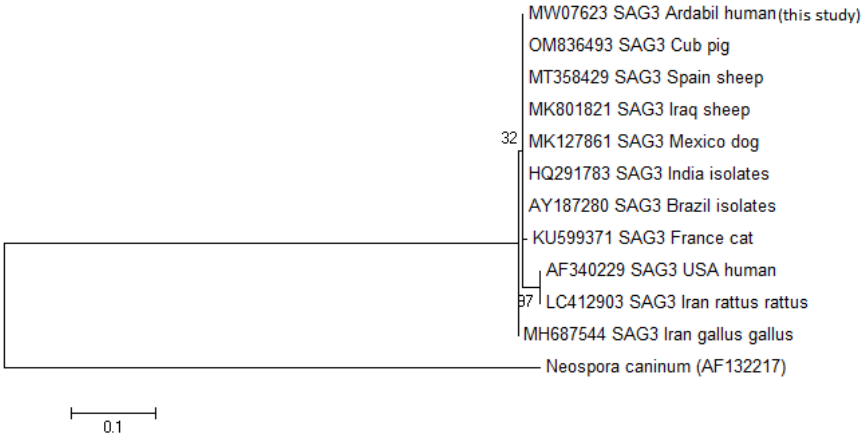

Fig. 3: Phylogenetic relationship of *T. gondii* isolates obtained in this study (accession NO. MW076233) and reference sequences retrieved from GenBank, using Tamura-Nei model of the Maximum Likelihood Method by MEGA6 based on the *SAG3* gene with *Neospora caninum* as the out-group

Table 1: Demographic characteristics, anti-*T. gondii* antibodies distribution, and logistic regression analyses of studied factors

Variable		IgG		IgM		Univariate		Multivariate	
		Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age groups	10–19	8 (3.8)	51 (24.3)	0 (0)	59 (28.1)	0.86 (0.8-0.9)	0.359	0.87 (0.85-0.9)	0.301
	20-29	20 (9.5)	69 (32.9)	2 (1)	87 (41.4)				0.919
	30-39	19 (9)	36 (17.1)	0 (0)	55 (26.2)				0.938
	40-49	2 (1)	5 (2.4)	0 (0)	7 (3.3)				-
	≥ 50	0 (0)	0 (0)	0 (0)	208 (99)				-
Educational levels	Illiterate	7 (3.3)	11 (5.2)	0 (0)	18 (8.6)	0.6 (0.56-0.64)	0.398	0.52 (0.5-0.6)	0.697
	High school	15 (7.1)	51 (24.3)	1 (0.5)	65 (31)				0.574
	Diploma	14 (6.7)	59 (28.1)	1 (0.5)	72 (34.3)				0.573
	Associate degree	5 (2.4)	15 (7.1)	0 (0)	20 (9.5)				0.692
	Bachelor degree	7 (3.3)	23 (11)	0 (0)	30 (14.3)				0.886
	Master's degree	1 (0.5)	2 (1)	0 (0)	3 (1.4)				-
Occupation	Employed	7 (3.3)	41 (19.5)	0 (0)	48 (23)	0.99 (0.9-1)	0.206	0.85 (0.8-0.9)	0.211
	Unemployed	42 (20)	120 (57)	2 (1)	160 (76.2)				
Residency	Rural	34 (16.2)	108 (51.4)	0 (0)	142 (67.6)	1.4 (1.4-1.5)	0.752	1.2 (1.1-1.3)	0.753
	Urban	15 (7.1)	53 (25.2)	2 (1)	66 (31.4)				
Contact with cat	Yes	16 (7.6)	46 (21.9)	0 (0)	62 (29.5)	1.1 (1.07-1.2)	0.421	0.9 (0.8-1.1)	0.426
	No	33 (15.7)	115 (54.8)	2 (1)	146 (69.5)				
Contact with soil	Yes	34 (1.2)	86 (41)	1(0.5)	119 (56.7)	1.3 (1.3-1.4)	*0.003	1 (0.9-1.1)	*0.006
	No	15 (7.1)	75 (35.7)	1(0.5)	89 (42.4)				
Raw or undercooked meat or liver consumption	Yes	26 (12.4)	66 (31.4)	0 (0)	92 (43.8)	1.2 (1.1-1.3)	0.126	0.95 (0.89-1.1)	0.132
	No	23 (11)	95 (45.2)	2 (1)	116 (55.2)				
Raw or unwashed vegetable consumption	Yes	46 (21.9)	31 (14.8)	2 (1)	75 (35.7)	1.1 (1.09-1.2)	*0.000	1 (0.9-1.2)	*0.000
	No	3 (1.4)	130 (61.9)	0 (0)	133 (63.3)				
Washing vegetable/fruit	Water	47 (22.4)	148 (70.5)	2 (1)	193 (91.9)	1.7 (1.6-1.76)	*0.050	1.5 (1.4-1.6)	*0.006
	Detergent	2 (1)	13 (6.2)	0 (0)	15 (7.1)				
Blood transfusion	Yes	4 (1.9)	5 (2.4)	0 (0)	9 (4.3)	0.9 (0.88-0.95)	0.448	0.7 (0.68-0.75)	0.446
	No	45 (21.4)	156 (74.3)	2 (1)	199 (94.8)				
Organ transplantation	Yes	2 (1)	0 (0)	0 (0)	2 (1)	0.88 (0.86-0.9)	0.715	-	-
	No	47 (22.4)	161 (76.7)	2 (1)	206 (98.1)				
History of abortion	Yes	12 (5.7)	35 (16.7)	0 (0)	47 (22.4)	1 (1-1.1)	0.730	0.8 (0.7-0.9)	0.732
	No	37 (17.6)	126 (60)	2 (1)	161 (76.7)				
Stillbirth	Yes	3 (1.4)	5 (2.4)	0 (0)	8 (3.8)	0.9 (0.88-0.95)	0.890	0.7 (0.7-0.8)	0.889
	No	46 (21.9)	156 (74.3)	2 (1)	200 (95.2)				
Stage of pregnancy	first trimester	38 (18.1)	121 (57.6)	2 (1)	157 (74.8)	1.5 (1.4-1.6)	*0.010	1.2 (1.1-1.3)	*0.001
	second trimester	10 (4.8)	22 (10.5)	0 (0)	32 (15.2)				0.016
	third trimester	1 (0.5)	18 (8.6)	0 (0)	19 (9)				0.011

*There was a significant association between these variables and seropositivity (*P*<0.05)

Table 2: *T. gondii* serological and molecular findings in pregnant women who had abortion during pregnancy

Age	NO. pregnancies	Month of abortion	NO. abortion	Contact/cat	Raw Vegetable	IgG		IgM		PCR-buffy coat	PCR-placenta	IgG Avidity
						Before abortion	After Abortion	Before abortion	After Abortion			
22	1	5	0	Yes	Yes	+	+	-	+	+	-	Low
41	2	2	1	NO	Yes	-	-	-	-	-	-	-
29	2	3	0	NO	Yes	+	+	-	-	-	-	High
17	1	4	0	NO	Yes	-	-	-	-	-	-	-
43	3	4	0	NO	NO	-	+	-	-	-	-	High
33	2	3	1	NO	Yes	-	-	-	-	-	-	-
25	2	5	0	NO	Yes	-	-	-	-	+	+	-
29	2	3	1	NO	Yes	+	+	-	-	-	-	High
20	1	2	0	NO	Yes	-	+	-	+	+	+	Low
39	2	1	0	NO	Yes	-	-	-	-	-	-	-

Discussion

Our findings showed that the overall prevalence of *anti-T. gondii* IgG antibodies in pregnant women was 24.3%, and only 1% of them were seropositive for IgM antibodies. *Toxoplasma* infection had a relatively high prevalence in Iranian pregnant women. In a meta-analysis, the seroprevalence of IgG anti-*T. gondii* antibody was detected at 32% (23). In contrast to these findings, this rate in Northern Iran was 43.8% (24), 44.8% in Ilam (25), and 37.2% in Zanjan Province (26). Anti-*Toxoplasma* IgG antibodies were identified in 22.1%, 27%, 20.25%, and 24.3% of Ardabil, Hormozgan, Alborz Province, and Arak City women, respectively, which are in accordance with the results of our study (27- 29).

Toxoplasma could be transmitted to the fetus even if the mother is infected several months before conception, and this could explain the low number of pregnant women who have IgM antibodies (30). In the present study, although 30% (3/10) of women with abortion were IgG-positive before abortion, the seroprevalence rate of IgG antibodies was 50% immediately after the spontaneous abortion. Interestingly, two women who tested positive for IgM antibodies in the initial screening had no abortion. On the other hand, two women who had abortions were positive for IgM after the abortion. To confirm the recent infection, a reliable method, the avidity test, was used. Low-avidity in our results increased the risk of *T. gondii* infection in the first three months of pregnancy. Of course, low-avidity antibodies may take months depending on the immune system and the development of IgG antibodies (31-34). Women suffering from chronic infections before pregnancy prevent reinfection during pregnancy and protect the fetus from vertical transmission (23). In other words, congenital toxoplasmosis occurs due to a recent and active infection of the mother. However, this paradigm has been recently discussed because of different strains circulating in Iran and the world at any time, *T. gondii* reinfection with a genetically distinct strain in an apparently "immunized" person is possible (35). In addition, the association of latent *Toxoplasma* infection with infertility or bad obstetric outcomes, slow fetal growth, and even poor motor skills in newborns after birth has been reported (36-39).

In this study, seven women had no history of abortion and three women had a previous abortion. Abortion occurred in the first trimester of pregnancy in six pregnant women and in the second trimester of pregnancy in four women. In the current study, the parasite's DNA was detected in the three placenta and two blood samples. All isolates were identified as genotype III. In previous studies in Iran, Hoveyda et al. (40) identified *T. gondii*

DNA in 15.48% of paraffin blocks obtained from products of conception. All positive cases belonged to genotype III of *T. gondii*, which is the most common genotype in the world and Iran (16, 41-43). However, in some studies genotype I (44, 45), and genotype II have been reported in various hosts (41, 46). Among *T. gondii* genotypes, type I has the highest levels of virulence and causes severe infection in humans (47). In a genotyping study in Shiraz, South of Iran, *T. gondii* types II and III were detected in 83.1% and 16.9% of women with spontaneous abortion, respectively (48). Ajzenberg et al. detected *T. gondii* type II in 85% of isolates associated with human congenital toxoplasmosis in France (49). In the study by Messaritakis et al., 20 isolates of *T. gondii* belonging to pregnant women in Cyprus 15 isolates were type III, and 5 isolates were type II (50).

Phylogenetic analysis showed that all of our *Toxoplasma* isolates were closely related to type III of *T. gondii* at the *SAG3* locus. Moreover, the findings revealed that the *SAG3* marker appropriately shows the 100% homologies between our isolates and other isolates from Iran and other countries.

Although in PCR-positive samples, the parasite's viability must be proven, the potential of the blood to cause infection in the fetus is confirmed. Therefore, the inclusion of screening tests, anti-*T. gondii* antibodies in routine prenatal care plans, and receiving timely diagnosis and treatment during pregnancy can affect rates of vertical transmission and congenital infections (51, 52).

There were a few inevitable limitations in this study. One important was the small sample size due to the coincidence with the COVID-19 pandemic, which led to pregnant women refusing to participate in the study. On the other hand, IgM antibody was not assessed in newborns simultaneously with mothers. Future follow-up studies at large sample sizes among pregnant women and newborns will be important for showing acute congenital toxoplasmosis and avoiding false-positive results.

Conclusion

The seroprevalence of *T. gondii* infection among pregnant women in the Meshkin-Shahr district is not high (24.3%). *T. gondii* type III was found in both buffy coat samples and placenta tissues as the etiology of fetus infection in mothers with spontaneous abortion. In addition, immunized pregnant women before pregnancy are not protected against congenital transmission, so screening during pregnancy is suggested for both seropositive and seronegative women. However, results should be interpreted with caution, and using molecular methods (real-time) that measure parasite loads seems to be essential.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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