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Serological tests and polymerase chain reaction for detection of Toxoplasma gondii infection in women attending for premarital examination

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Hawre Mustafa Bakre *

Abstract

Background and objective: Human infection with toxoplasmosis is common and is usually asymptomatic, and congenital form is one of the most important clinical aspects of this disease. This study aimed to determine anti-toxoplasma antibodies, and *Toxoplasma* DNA in premarital women referred to Mammon Dabax health centers and to design prevention policies after marriage and during their pregnancy.

Methods: One hundred fifty premarital women who were examined for pre-marriage laboratory testing were enrolled in this cross-sectional study. Blood samples were tested for specific anti-toxoplasma IgM and IgG antibodies using an enzyme-linked immuno-sorbent assay (ELISA) and detection of B1 gene of *T. gondii* by PCR.

Results: Of 150 sera examined of premarital female 35 (23.3%) were seropositive for anti-toxoplasma antibodies by latex agglutination test. Using ELISA test, 8 (5.3%) of the sera examined were seropositive for anti-toxoplasma IgM; meanwhile, 23 (15.3%) sera were positive for anti-toxoplasma IgG. The ELISA test finding for anti-toxoplasma IgM and IgG of the total sera examined were subsequently subjected to PCR. Thus, PCR analysis for detecting *T. gondii* DNA in the blood of premarital female was positive in 15 (10%) of the total DNA samples. Of these 15 positive PCR when correlated with positive ELISA finding 7 (46.6%) and 4 (26.6%) blood samples were positive for anti-toxoplasma IgM and anti-toxoplasma IgG, respectively.

Conclusion: PCR assay has an advantage in the detection of recent and active infections as reflection or marker for *T.gondii* parasitemia. ELISA can be used as a highly sensitive screening test while IgM anti-toxoplasma antibody positive is not enough to confirm recent infection as the false negative is high.

Keywords: Toxoplasma gondii; Premarital female ELISA; PCR; toxoplasmosis.

Introduction

Toxoplasma gondii is a ubiquitous apicomplexan parasites of human and others warm-blooded animals which have been considered as the cause of the most prevalent parasitic zoonosis[1,2] and is the causal agent of significant morbidity and mortality among human worldwide.[3,4] In general, *T. gondii* infections are asymptomatic, and self- limiting, especially among healthy immunocompetent hosts; however the infection may cause severe complications in pregnant women and immunocompromised patients.[5,6] Fetal toxoplasmosis, particularly in early pregnancy can cause miscarriage, stillbirth, and birth defects.[7] Early first-trimester maternal infection is less likely to result in congenital infection, but the squeal is more severe.[8,9] The detection of recently acquired infection in pregnant women is, therefore, critical for clinical management of the mother and her fetus.[10] The diagnosis of toxoplasmosis is routinely based on serological tests for the presence of immunoglobulin IgM and IgG-specific antibodies to *Toxoplasma* by enzyme-linked immunosorbent assay (ELISA).[11] These tests, however, are not ideal because antibody production either fails or is significantly delayed. Most cases of active toxoplasmosis are due to

* Department of Microbiology, College of Medicine, Hawler Medical University, Erbil, Iraq.

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reactivation of latent infections, which is why direct demonstration of the parasite in tissues or other fluids by polymerase chain reaction (PCR) assay is a major breakthrough for the diagnosis of toxoplasmosis in these patients.[12,13] PCR was first developed for the diagnosis of congenital toxoplasmosis in amniotic fluid.[14] The detection of *T. gondii* DNA in blood has highlighted the possibility of anticipating the diagnosis compared with radiological findings and histology. PCR assay is an important technique to evaluate the prevalence of *Toxoplasma* reactivation when the detection of circulating DNA is the only clue to its reactivation.[15] Recently, researchers[16,17] illustrated that the serological diagnosis of recent infection in early pregnancy could be confirmed by a positive *Toxoplasma*-specific PCR result from blood samples in the first half of pregnancy. However, the reliability of the PCR is a crucial technique to evaluate the prevalence of *Toxoplasma* reactivation when the detection of circulating DNA is the only clue to its reactivation.[18,19] B1 gene is highly conserved in all *T.gondii* strains.[20] Approximately 35 copies of this gene are presented in the *T. gondii* genome, but absent in the other mammalian cells.[21] Thus, this study aimed to determinate the relationship between serological and molecular methods for detection of *T. gondii* in the blood of premarital female and measure the sensitivity and specificity of ELISA for anti-toxoplasma (IgM and IgG) in comparison to test by PCR technique.

Methods

Subjects:

This cross-sectional study was carried out from January to September 2015. The study was conducted on 150 premarital women attending Mammon Dabax health centers. The samples were collected from pre-married women from Mammon Dabax counseling health center and processed at the Microbiology Department in the College of Medicine, Hawler Medical University. The sera were tested for *T. gondii* antibodies (IgG and IgM) and confirmed by PCR for *T.gondii* infection. Informed consent was obtained, and then blood samples were withdrawn. History including age, residency, contact with cats, family history of abortion and type of blood group were taken for each of the enrolled premarital female.

Sample collection

A 5 mL venous blood sample was collected from all screened women. Serum was separated from half of each sample and kept at –20 °C until used, while the other half of the sample was placed in a sterilized EDTA tube and stored at –80 °C for amplification by PCR. The entire study group was screened for *Toxoplasma* infection with a rapid latex agglutination test (Latex-Toxo kit, Biokit Company) and ELISA kits (Biokit Diagnostics Company, Spain) according to the manufacturer's instructions.

Methods

A- Latex agglutination test (LAT):

Toxocell LATEX is a one step rapid LATEX particle agglutination test or slide for qualitative and semi-quantitative determination of *Toxoplasma* antibodies in serum. Qualitative Test and Semi-quantitative Test: the serum samples are reacted directly with *Toxoplasma* antigens for the qualitative test by using Toxocell LATEX (bio-kit company Linear Chemicals Barcelona, Spain).

B-Determination of IgM and IgG by ELISA:

ELISA assay: The collected serum samples from premarital female were tested for the presence of the specific IgM and IgG antibodies by using Clinotech Toxo ELISA IgM and IgG kits (Biokit Diagnostics Company, Spain). Clinotech Toxo IgM and IgG ELISA kits are microwell ELISA test designed for the qualitative detection of IgM or IgG antibodies to *T.gondii* in human serum. ELISA in small ruminants: ELISA was carried out according to manufacturer instruction as described by.[22]

Confirmatory testing for *T. gondii* by PCR:

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C-DNA

Extraction DNA was extracted from whole blood samples, using DNA extraction kit (High Pure PCR Template Preparation Kit) by Roche Company, according to the manufacture protocol. Polymerase chain reaction was carried out according.[21] The purity of DNA extracted from whole blood samples was measured by nanodrop and kept at-70 □C until PCR was performed.

PCR assay:

The specific primers TR1 (5-ACGAACACTCGCAGAGATGA-3) and TR2(5-GATCCTTTTGCACGGTTGTT-3) was designed for B1 Gene. PCR was performed in a final volume of 25 µl by adding 12.5 µl of Fast start PCR master mix is ready –to - use.2x concentrated master mix that contains all the reagents (except primers and DNA template) needed for running PCR reactions on thermal block cycler instruments, FastStart Tag DNA polymerase, magnesium chlorid, double concentrated reaction buffer and nucleotides(dATP, dCTP, dGTP, dTTP, 0.4mM each). 3 µl DNA template and 1.25 µl of primers at a concentration of 1 pM,and 7 µl ddH2O. Each of 35cycles of PCR thermal was consisted of an initial denaturation cycle for 3min at 94□C, denaturation of DNA for 30 seconds at 94□C, annealing for 30 seconds at 45□C, extension for min at 72□C. 15 µl of the amplified PCR product was analyzed on 1.5% agarose gel electrophoresis and visualized under transluminat or after Ethidium bromide staining. Every PCR run included positive and negative controls. Deionized water was used as negative control, and positive *Toxoplasma* sample which provided in private laboratory was used as positive control.

Statistical analysis: All statistical analyses were performed using the statistical package for the social sciences (version 17). The frequencies and percentages were presented and the $\chi 2$ test was used for statistical evaluation of the categorical variable. McNemar test was used for testing sensitivity and specificity of ELISA test. A *P* value of ≤0.05 was considered to be statistically significant.

Ethical considerations: The study was approved by the Research Ethical Committee of the College of Medicine, Hawler Medical University, Erbil. A written informed was obtained from each participant before collection of samples.

Results

Serologic and PCR findings of the 150 samples screened for anti-toxoplasma antibodies and DNA for premarital female are shown in Table 1. Thus latex agglutination test was positive in 23.3% of the samples. Meanwhile, anti-toxoplasma IgG and IgM using ELISA were seropositive in 15.3% and 5.3%, respectively. PCR was positive in 10% of the blood screened for *T. gondii* DNA organism

Table 1: Average results of LAT total immunoglobulin tests, ELISA IgM and IgG immunoglobulin and PCR for detecting both anti-toxoplasma antibodies and DNA in 150 samples of premarital females.

Tests	Positive samples		Negative samples		Total
	No.	%	No.	%	
Latex	35	(23.3%)	115	(76.7%)	150
ELISA IgG	23	(15.3%)	127	(84.7%)	150
ELISA IgM	8	(5.3%)	142	(94.7%)	150
PCR	15	(10%)	135	(90%)	150

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The serostatus of anti-toxoplasma antibodies among premarital women of different age groups using latex agglutination, ELISA IgG and IgM and PCR finding is shown in Table 2. The age group <20 years showed the highest degree of seropositivity than the other age groups, but there was no statistically significant difference among different age groups.

Besides the serological testing for anti-toxoplasma antibodies, PCR technique was used to confirm the infection with *T. gondii* by detection of *T. gondii* DNA in the blood of premarital female. *Toxoplasma gondii* DNA was successfully extracted and analyzed by PCR. Thus, 15 samples were positive by PCR.

Table 2: Distribution of anti-toxoplasma antibodies and toxoplasma antigen in relation to age groups.

Age	Latex		IgM		IgG		PCR	
	+Ve (%)	-Ve (%)	+Ve (%)	-Ve (%)	+Ve (%)	-Ve(%)	+Ve(%)	-Ve (%)
<20 No. (69)	19 (27.5%)	50 (72.5%)	7 (10.1%)	62 (89.9%)	13 (18.8%)	56 (81.2%)	11 (15.9%)	58 (84.1%)
20-24 No.(15)	2 (13.3%)	13 (86.7%)	1(6.7%)	14 (93.3%)	3(20.%)	12(80%)	1 (6.7%)	14 (93.3%)
25-29 No.(52)	13 (25%)	39(75%)	----	52 (100%)	4(7.7%)	48 (92.3%)	2(3.8%)	50 (96.2%)
≥30 No. (14)	1(7.1%)	13 (92.9%)	-----	14 (100%)	3 (21.4%)	11 (78.6%)	1(7.1%)	13 (92.9%)
Total	35 (23.3%)	115 (76.6%)	8(5.3%)	142 (94.7%)	23 (15.3%)	127 (84.7%)	15 (10.0%)	150 (100%)
X^2	3.652		6.935		3.645		5.207	
P value	0.302		0.074		0.302		0.157	

Table 3: Positive and negative finding of T. gondii infection by PCR among examined premarital women.

PCR	Number	%
Positive	15	10
Negative	135	90
Total	150	100

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Positive samples were detected by presenting a 120 bp band of amplification test on 1.5% agarose gel (Figure 1). The association between ELISA IgG, IgM serostatus and PCR is shown in Table 4. Among the total 15 PCR positive for *T.gondii*, 4 (26.6%) and 7 (5.1%) were seropositive for anti-toxoplasma antibodies IgG and IgM, respectively. Sensitivity and specificity of ELISA were measured according to the gold diagnosis standard of *T. gondii* (i.e. PCR). The studies showed that the sensitivity and specificity of ELISA in detecting anti-toxoplasma IgM, IgG were 46.66%, 26.66% and 99.29%, 85.93%, respectively. Statistically, the relation between anti-toxoplasma antibodies IgG, IgM and PCR were significant and highly significant, respectively.

Table 4: The association between ELISA- IgG, IgM and PCR test in study group.

PCR	ELISA-IgM				ELISA-IgG			
	No. +Ve	(%)	No. -Ve	(%)	+Ve	(%)	-Ve	(%)
Positive No. (15)	7	(46.6%)	8	(53.33%)	4	(26.6%)	11	(73.33%)
Negative No. (135)	1	(0.74%)	134	(99.26%)	19	(14.07%)	116	(85.93%)
Total (150)	8	(5.33%)	142	(94.67%)	23	(15.33%)	127	(84.67%)
X^2	**7.1**				**4.1**			
***P* value**	**0.008**				**0.041**			
Sensitivity	7/15*100=46.66%				Sensitivity	4/15*100=26.66%		
Specificity	134/135*100=99.25%				Specificity	116/135*100=85.93%		
PPV+	7/8*100=87.5				PPV+	4/23*100=17.4%		
NPV-	116/142*100=94.36				NPV-	116/127*100=91.33%		

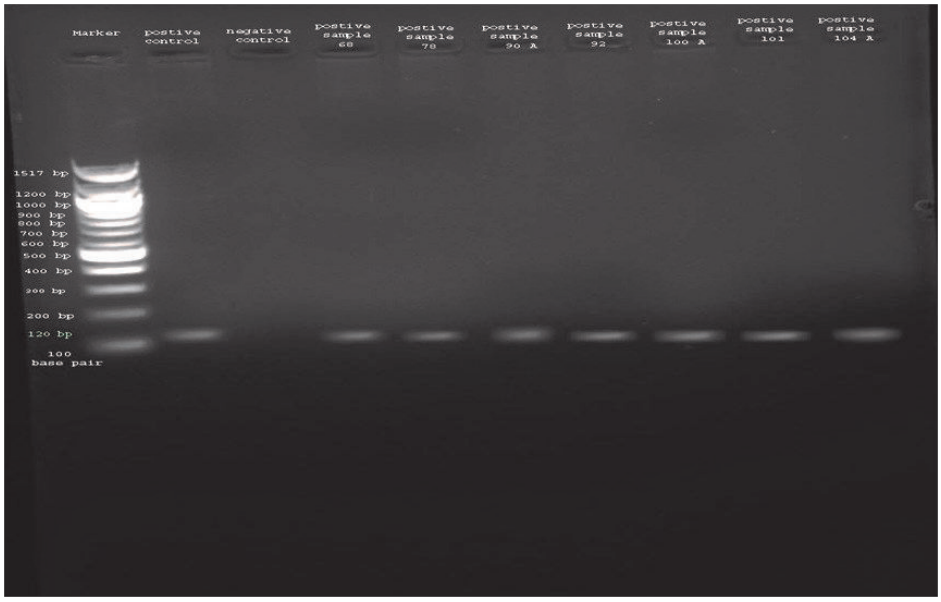

Figure 1: PCR amplification of the B1 gene of *Toxoplasma gondii* genome. Lane 1. Marker, Mol. wt. marker (100 bp ladder), lane 2 positive control, lane 3 negative control, lane 4, 5, 6, 7, 8, 9, and 10 are positive samples.

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Discussion

Toxoplasmosis is an infection caused by an intracellular protozoan called *T. gondii*. Although most cases of this infection in immunocompetent individuals are asymptomatic but in pregnant women, it can cause congenital toxoplasmosis with severe pathological effects on fetus.[23,24] Congenital toxoplasmosis is a major problem in most communities with a high prevalence of *T. gondii* infection and study of the seroepidemiology of this infection among women of childbearing age could provide appropriate approaches to design the preventive measures.[25] Thus, the aim of the present study was to determine the relationship between serological and molecular methods for detection of anti-toxoplasma antibodies and *T.gondii* organism in the blood of premarital female referring to Mammon Dabax health centers before marriage in Erbil city. In the present study, LAT results were not accurate enough for interpretation or diagnostic purposes. Wherever, LAT test principally depends on detection of total immunoglobulin via naked eye. Thus differ from one person to another; LAT was detecting higher seropositive results compared to ELISA and PCR (23.3% versus 15.3% and 10%). The results were consistent with numerous studies that comparing LAT and ELIAS.[26,27] In the present study, ELISA results showed that 23 out of 150 cases (15.3%) and 8 cases (5.3%) were positive for specific IgG and IgM anti-toxoplasma antibodies respectively. Our findings are similar that stated by[28,29] regarding the prevalence of total toxoplasma antibodies in people applicants for marriage in the Babylon province by use ELISA test while the results of our study were disagreement with some studies regarding the percent of infection.[30-32] Regional variations in the incidence of *Toxoplasma* infection rates from one country to another or even within the same country has been well documented.[33] This variation has been attributed to various factors such as living location, climate, age, host immunity, genus, and genotype of parasite and cultural differences regarding hygienic and feeding habits.[34-36] These results refer the importance of *Toxoplasma* test before marriage. The highest percentage of infection with toxoplasmosis by ELISA for IgG in the serum sample of premarital women was seen in 20-24 year age as (20%). This disagrees with that studied by[37] who reported anti-toxoplasma, IgG of 37.5% among age group 42-and more; 30% among 40-49 years;[38] 85.39%, among 41-50 years;[49] 66% among 25-29 years.[40] ELISA-IgM was positive in 10.1% of sera examined among age group <20 years this finding disagreed with[41] showed findings with IgM anti-toxoplasma was 30% among the age group 18-25 years; other reported 30% IgM anti-toxoplasma seroprevalence among the age group 18-25 years.[42] PCR in the blood of premarital women according to age group the higher among age group <20 years was 15.9% The present study disagreed with the studies by,[43,44] which they found high signification among the age group 39-43 years was 52.1%. These differences between previous results and the current result given the differences in the specificity and sensitivity of method used for diagnosis and response of every host to the strain of parasite, the differences in parasite strains may play a major role in the stimulation of host immune response against the parasite.[45] The present result might be relevant to the different number of each infected person at each age group. Also, the peoples may be contacted with *Toxoplasma* in childhood, through cats connected, soil exposure has resulted in accumulating of anti Toxoplasma antibodies at different percentages within the human being that lead to the chronic infection with toxoplasmosis.[46] The results of the present study confirm the occurrence of considerable percentages of true *T. gondii* infection in the premarital female. All positive blood samples by LAT, ELISA (IgG and IgM) tests collected from

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premarital female showed only 15 positive samples by PCR technique based TaqMan probe for detection of *T. gon di*s which represented (10%). This result agrees with a number of studies has already shown that a positive PCR result is not always accompanied by positive serology indicating the local synthesis of antibodies[47,48] while the results of our study were in agreement with the results of other studies.[49,50] Polymerase chain reaction has been advised to overcome false-negative serologic testing, particularly in compromised immune individuals.[51] The diagnostic value of PCR for the detection of *T. gondii* in blood samples has been evaluated from both immune-competent and compromised patients.[52,53] Several PCR-based techniques have been developed as alternative diagnostic measurements for *T. gondii* infection. These techniques make use of the most conserved gene sequences among different strains of *T. gondii*, including the B1 gene repetitive sequence, the P30 (SAG1) gene, and ribosomal DNA.[54] The use of the B1 gene for *T. gondii* detection originated with,[55] who combined PCR amplification with Southern blotting to detect a specific B1 gene product. Although serological testing has been one of the primary diagnostic approaches for toxoplasmosis, it has many limitations; it may fail to detect specific anti-Toxoplasma IgM or IgG during the active phase of infection because these antibodies may not be produced until after several weeks of parasitemia. Furthermore, the test may fail to detect *T. gondii* infection in certain immune-compromised patients because the titers of specific anti-Toxoplasma antibodies may fail to rise in this type of patient.[55] Indeed detection of *T. gondii* DNA using PCR minimizes the problems faced when using serodiagnostic assays. Therefore, the negative results obtained by both PCR and ELISA rule out an infection in premarital women. The fact that could explain the proportion of 0.74 % of premarital women that revealed negative PCR results and positive IgM is the probability to find women with residual IgM detected during prolonged periods of time. *Toxoplasma* DNA was found in 26.6% premarital women with positive IgG indicates chronic infection since it is known that women with latent toxoplasmosis present intermittent parasitemia with low parasite burden.[56] *Toxoplasma* DNA was detected in 73.33% seronegative premarital women; it could correspond to a very recent infection at the time of serological leading to an insufficient production of immunoglobulin not detected by serology, or other explanation that those patients are not able to produce specific antibodies, representing a state of immunodeficiency.[55] Molecular tests that could detect the presence of circulating parasites would be of extreme application in this scenario. A positive serological result is only indicative of exposure, whereas direct detection of *T. gondii* in blood or other clinical samples categorically confirms the parasite presence leading to the diagnosis of primary, reactivated or chronic toxoplasmosis.[57] The PCR-based method described in this study provides a rapid, sensitive, and quantitative way of detecting *T. gondii* in clinical specimens.

Conclusion

In conclusion, PCR amplification of the B1 gene of *T.gondii* using whole blood is rapid, sensitive and specific diagnostic procedure and considered a valuable tool for establishing the diagnosis of *T.gondii* infection in adult females before or during pregnancy. The serological immune profile is heterogeneous and may be delayed. This makes it an unreliable method for diagnosis and/or treatment follow-up. Thus, it is advisable to rely on PCR to detect *T.gondii* DNA for diagnosis and monitoring of infection during treatment.

Conflicts of interest

The author reports no conflicts of interest.

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