Annals of Parasitology 2021, 67(4), 733–740
doi: 10.17420/ap6704.390

Original paper

Efficiency of molecular detection of *Toxoplasma gondii* contrasting to serological method

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ABSTRACT. Toxoplasmosis infection of the mother during pregnancy is linked to transplacental transmission to the fetus. *Toxoplasma gondii* infection is difficult to diagnose and necessitates a comprehensive examination that includes laboratory and clinical testing. This study used RT-PCR using B1 gene based on paired blood samples and clinical data, for the detection of *Toxoplasma* infections in miscarriage women, this technique was compared to enzyme-linked immunosorbent assay (ELISA). There are a total of 125 miscarriage women at Al-Muthanna Hospital. Anti-*Toxoplasma* antibodies, IgG (latent infection) and IgM (recently acquired infection), were positive in 50/125 cases, with 25.6% IgG, 8.8% IgM, and 5.6% for IgM and IgG. In addition, women who have had two miscarriages have a high seropositivity rate. Furthermore, the development of molecular technologies to amplify parasite nucleic acids has enhanced toxoplasmosis diagnosis. The cycle threshold values C_t, which indicate the target gene's quantity, were calculated. With DNA from 500 *T. gondii* tachyzoites, a C_t of 25–28 was usually obtained. Present results show that the assay's reproducibility was found in 50 which considered (40%) from total miscarriage women of ELISA positive samples while 28/125 samples (22.4%) will positively in molecular detection and 56% from serological test was positive in molecular test. A move forward was the invention of diagnostic methods that used ELISA IgG and IgM followed by an RT-PCR methods in women were the most effective in detecting recent and reactive *Toxoplasma gondii* infection.

Keywords: ELISA, molecular, miscarriage, IgG, IgM

Introduction

The transmission of *T. gondii* to the fetus is often linked to primary maternal infection during pregnancy. *T. gondii* infection during pregnancy can result in serious congenital infection, including abortion [1]. Congenital toxoplasmosis is the most serious manifestation of infection resulting from the vertical transmission of *T. gondii* transplacentally from a parasitemic mother to her offspring. The severity of disease depends on the gestational age at transmission [2]. In addition, to the trimester of maternal infection was acquired. For untreated women, the rate of transmission is nearly 25%, 54%, 65% respectively [3]. However, approximately 50% of frequent miscarriages are not attributed to these etiological factors and their causes remain unknown [4]. However, 13 million HIV-infected people were *Toxoplasma*-seropositive worldwide, thus at risk for cerebral toxoplasmosis, with 87% of them living in sub-Saharan Africa [5]. In Arab world including Iraq, toxoplasmosis has high prevalence rate of infection in pregnancy patients [6]. The acute infections in pregnant women can lead to congenital toxoplasmosis, which may cause blindness, mental retardation, or even death of the fetus [7]. Here, the study demonstrates specificity of toxoplasmosis detection in a miscarriage women and associated with congenital toxoplasmosis initially by using serological diagnosis by enzyme-linked immunosorbent assay (ELISA) method [8]. In addition to the molecular diagnosis is an essential, accurate tool for the diagnosis of congenital toxoplasmosis and to evaluate the prevalence of *Toxoplasma* reactivation when the detection of circulating DNA is the only clue to its reactivation and its effect on gene expression in immune cytokines [9]. The aim of this study is to analyze and describe a possible diagnostic tool.

Materials and Methods

Study design

A total of 125 Iraq women suffering from single or recurrent miscarriage attend delivery's hospital in Al-Muthanna governance in the period between January to July 2019. Addition to 50 healthy women were used as a control. This study was carried out on women ranging in age from 20–44 years. Women suffering from immunosuppressive diseases or systematic diseases were excluded from the study because they affect dissemination of samples and may interfere with association of toxoplasmosis disease with genotype status. Venous blood samples (6 ml) were collected from each woman and distributed into three tubes, the first two heparin tubes were used for immunological and the third tube used for molecular analysis.

Detection of Toxoplasma (*IgG, IgM*) *by ELISA technique*

The *Toxoplasma* IgG ELISA kit was used for qualitative and quantities detection of IgG antibodies to *T. gondii* in serum samples, according to the procedure of Call Biotech Mini Kit, USA (Toxoplasma; IgG, IgM) ELISA Kit. The results are read by a micro well reader associated in a parallel manner by calibrator and controls [10]. Optical density (450 nm) was measured with micro ELISA reader according these equations: Cut-off value = Calibrator O.D. × Calibrator factor (CF); Control (–ve) = Control negative O.D./Cut-off value; Control (+ve) = Control positive O.D./Cut-off value and Ab index = Sample O.D./Cut-off value.

DNA extraction

Genomic DNA was extracted from EDTA-coated venous blood samples collected from each case study using (Favor Prep Genomic DNA) Mini Kit following the manufacturer's instructions for DNA purification from blood. Final pellets were re suspended in 30 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.2) and stored at –20°C until used. DNA solution was diluted to 100 µl with elution buffer and kept at −20°C before further molecular analysis. Quantity of *T. gondii* DNA was quantified according to [11] by amplification of B1 gene by RT-PCR using specific primers:

F-5'-AAGCAGCGTATTGTCGAGTAGAT-3'; R'-5CGTCTCTTTCATTCCCACATTTT-3' under the following thermal cycling conditions Go Taq DNA Polymerase activation 1 cycle for 5 min at 95°C, initial denaturation for 1 cycle at 95°C for 5 min, denaturation for 40 cycle at 95°C, then primer annealing and extension for 40 cycle at 72°C for 2 min. and final extension at 72°C for 10 min.

Statistical analysis

All data were subjected to statistical analysis using SPSS win statistical package software version 13.0 for Microsoft Windows. Whereas the odds ratio (OR) was estimated for evaluating the risk related to age and resident; it is calculated by Chi-square, and Fischer's exact probability via utilizing the statistical software epidemiological (WINPEPI) version 11.65. Also, *P*-values are statistically significant when less than (0.05).

Ethical consideration

The studies were approved by Committee of Research, Publications and Ethics of the College of Biotechnology, Al-Nahrin University. All procedures were explained to patients in the local, and written or thumb-printed informed approval was gained.

Results and Discussion

A total of 125 women with single or multiple miscarriage in addition to 50 normal healthy were enrolled in this study as shown in table 1.

Toxoplasmosis women were classified into five age groups from 20–44 with four years' intervals results. In this study, the serological focused on distribution of antibodies type according many risk factors. The applied genetic study determines the presence of a specific genetic polymorphism that would contribute to further advancement of the disease in the presence of local etiological factors.

Seroprevalence of toxoplasmosis using ELISA IgM/IgG tests

The use of serologic tests for demonstration of specific antibody to *T. gondii* is the initial and primary method of diagnosis [12]. In the present study, seropositivity for IgG and IgM antibodies is used to diagnose toxoplasmosis, results illustrated in figure 1 that shows 8.8% and 25.6% of miscarriage women were positive for IgG and IgM respectively, where the serum levels are more than maximum levels recommended for these two antibodies more than 8 IU/l which indicates a positive infection with *T. gondii*. While the levels of these antibodies in normal healthy control women were 6.3 and 5.4 IU/l respectively are within the

Table 1. Age distribution of study groups including patients and healthy controls

Age group (year)	Miscarriage women		Healthy women	
	No.	%	No.	%
20–24	30	24*	20	40*
25–29	20	16**	10	20
30–34	25	20*	10	20
35–39	40	32	10	20
40–44	10	8	0	0

*P<0.05; **P≤0.01

Table 2. Seropositivity rate of *Toxoplasma* IgG among miscarriage women in relation with age groups

Age group (year)	No. (%)	IgG– No. (%)	IgG+ No. (%)	IgG prevalence (%)
20–24	30 (24)	23 (66.6)	7 (33.33)	21.8
25–29	20 (16)	11 (55)	9 (45)	28.12
30–34	25 (20)	16 (69)	9 (36)	28.12
35–39	40 (32)	34 (85)	6 (15)	18.7
40–44	10 (8)	9 (90)	1 (10)	3.125
Total	125	93 (74.4)	32 (25.6)	100
P-value≤0.001, X2=15.6		0.001*	0.001	

normal levels. These results indicate acute and chronic infection within the first trimester and were similar to [13].

Distribution of toxoplasmosis according to age

Distribution of toxoplasmosis among age groups was studied according to positivity of IgG and IgM antibodies. Results indicated in table 1 showed that high prevalence of toxoplasmosis occurred in miscarriage women with age group between 20 to 24 years, with a significant increase (P<0.05) compared with the prevalence of toxoplasmosis in other groups of miscarriage women.

In contrast, in other study, they found that the prevalence of toxoplasmosis increases with growing age of miscarriage women [14]. Furthermore, they found that the highest seroprevalence of infections with toxoplasmosis was among pregnant women in the 35–39 age group. Another study conducted by [15] who mentioned that the (26–30) years of age

Table 3. Seropostivity rate of *Toxoplasma* IgM among miscarriage women according to age of study groups including patients and healthy controls

Age group (year)	No. (%)	IgM+ No. (%)	IgM– No. (%)	IgM prevalence (%)
20–24	30 (24)	4 (13.33)	26 (76.77)	36.6
25–29	20 (16)	2 (10)	18 (90)	18.1
30–34	25 (20)	2 (8)	23 (92)	18.1
35–39	40 (24)	1 (3.33)	39 (97.7)	9.01
40–44	10 (8)	2 (20)	8 (20)	18.8
Total	125	11 (8.8)	114 (91.2)	100

P-value≤0.0001, X2=15.8

Table 4. The prevalence of toxoplasmosis IgM and IgG among miscarriage women

Age group (year)	Women tested No. (%)	IgM+ IgG+ No. (%)	IgM– IgG– No. (%)	IgM, IgG prevalence (%)
20–24	30 (24)	3 (10)**	27 (90)	42.8
25–29	20 (16)	2 (10)*	18 (90)	28.5
30–34	25 (20)	0	25 (20)	–
35–39	30 (24)	2 (10)	28 (93.3)	28.5
40–44	10 (8)	0	10 (8)	–
Total	125	7 (5.6)	118 (94.4)	100
P-value≤0.0001, X2=20.3		0.0001**	0.0001	

had a higher percentage of toxoplasmosis reported in women, which was inconsistent with current findings, while it agrees with [16] who reported that in (20–29) years age groups had lowest rate of infection with *T. gondii* (28.6%) in compare with other age groups. The present study showed that the seroprevalence *T. gondii* infection among the high risk pregnancy group was 28.12% for IgG compared with other groups as shown in table 2, and for IgM as shown in table 3. Other study recorded that positivity for IgG and IgM was 31.5% and 7.6% respectively [17], While seropositive for anti-*Toxoplasma* IgG and IgM, of 500 pregnant women was 145 (29%) and 7 (1.8%) respectively [18] (Tab. 4). High prevalence values of infection with *T. gondii* were found in young adult women, this probably happened due to more frequent *Toxoplasma* contact in childhood and adolescence, through cat's contact, soil exposure [19]. This variation depends on sample type and diagnosis method. In addition to the differences in the specificity and sensitivity of method used for diagnosis and response of host to the strain of parasite. *T. gondii* infection may be induces stable polytypic parasite-specific response characterized by high concentrations of but not IgG1 antibodies, which have been confirmed in mice [20].

In Babylon governorate in double frequent miscarriage women, levels of IgM and IgG were 4% and 22.6% [21]. Other study conducted by [22] founded that IgG positive cases were 112 (31.5%), IgM positive cases were 27 (7.6%) and both IgM and IgG positive cases were 9 women (2.5%). Furthermore, another study carried in Egypt about threatened miscarriage of pregnant women founded that prevalence of *T. gondii* was (18.3%, 38.3% and 18.3%) for (IgM, IgG, IgM and IgG) respectively [23]. On the other hand, results showed that the most common infection with *T. gondii* are with two birth. These findings are similar to [24] who stated that there is a significant relationship between women suffering from recurrent spontaneous miscarriage and two child birth.

Distribution of toxoplasmosis according to duration of pregnancy and miscarriage type

In this study, the period of miscarriage was graded according to the time of pregnancy in the first, second and third trimester. Results illustrated showed that the outcome of anti-*Toxoplasma* was significantly higher ($P<0.05$) in the first trimester than in second trimester. As the percentage of positive results for IgM+, IgG+ and IgM+ IgG+ in the first and second trimester were significantly higher ($P<0.05$) than in third trimester with the miscarriage period and infection with *T. gondii* (Tab. 4).

Results also showed that there is a statistical association ($P<0.05$) between the time of miscarriage and toxoplasmosis infection in patient groups. In the first trimester, wherever the anti-*Toxoplasma* outcome provides a high degree of toxoplasmosis than in a other period. It agree with [25] who mentioned that the anti-*Toxoplasma* IgG was higher in the first trimester than in other trimesters. This refers to the fact that pregnancy injury depends on the degree of resistance of the fetus as well as the immune system acquired spontaneously via the placenta, so that the fetus is more susceptible to infection during the first trimester of the non-immune system [26]. These results disagree with other study mentioned that miscarriage women with a single miscarriage have higher levels of anti-*Toxoplasma* antibodies compared to other patient groups (double and multiple abortion) in pregnant and non-pregnant

Table 5. Number and percentage distribution of accused by selected demographic characteristics (N=125)

Variable	Category	Positive case No. (%)	Negative case No. (%)	OR (95% Cl)
Miscarriage women	Single	19 (38)	50 (66.6)	0.36 (0.14–0.654) *P*-value=0.001
	Recurrent	31 (62)	25 (33.3)	
Residence area	Urban	22 (33.8)	51 (68)	2.70 (1.21–6.062) *P*-value=0.009
	Rural	28 (66.1)	24 (32)	
Education	No education	38 (76)	50 (66.6)	3.16 (2.29–4.64) *P*-value=0.238
	High education	12 (24)	25 (33.3)	
Occupation	No job	26 (52)	49 (65)	1.26 (0.62–2.55) *P*-value=0.478
	–	24 (48)	36(35)	
Fever	High fever	35 (70)	38 (50.6)	0.58 (0.28–1.21) *P*-value=0.147
No symptoms	–	15 (30)	47 (62.6)	
Type of delivery	Normal delivery	30 (60)	40 (53.3)	0.58 (0.29–1.20) *P*-value=0.175
	Caesarean births	20 (40)	10 (46.6)	

women [27]. 73% of the recurrent miscarriage have displayed a significant relationship between increase of positive-IgG level, and high level of LHL. Results indicated is a significant increase (P<0.05) in anti-*Toxoplasma* antibodies in recurrent miscarriage women compared with a single miscarriage (36%, 24%) results also showed that the total percentage of toxoplasmosis (40%) was higher than those conducted in other regions (9.4%) in Saudia Arabia [28]. The explanation of this variation may be due to variable sample size and ecological parameters.

However, it is becoming increasingly clear that rates of toxoplasmosis vary greatly between distinct populations. Results show in table 5 the prevalence of *T. gondii* infection was significantly higher (P<0.05) in rural area (70%) compared with urban (30%), which may be related to the increased people rate and pollution in urban compared with rural communities as the people in urban were highly contact with sources of infection than rural [29]. On the basis of this result, living in a rural area was found to be the only independent interpreter of toxoplasmosis (OR=2.70, CI=1.21–6.062, P=0.009) (Tab. 5). Etiological screening for anti-*Toxoplasma* antibodies should be routine tests especially among high-risk pregnant women. This may be concerning to mode of transmission of *Toxoplasma*. There were no differences in prevalence of toxoplasmosis based on employment or level of education.

Table 6. Rate of IgM and IgG antibodies against *Toxoplasma* corresponding the result of PCR in miscarriage women

Detection by ELISA		Detection by RT-PCR	
Seropositivity	No.	+ve result No. (%)	–ve result No. (%)
IgM+ IgG+	7	6 (21.4)	1 (4.5)
IgM+ IgG–	11	8 (28.5)	3 (13.6)
IgM– IgG+	32	14 (50)	18 (81.8)
IgM– IgG–	–	0 (0)	0 (0)
Total	125	28	22
P-value=0.019			

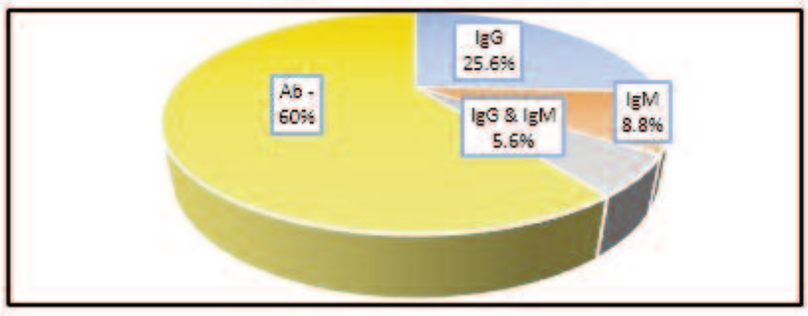

Figure 1. Percentage of toxoplasmosis antibodies in miscarriage women enrolled in this study

Isolation of genomic DNA

The purity of the DNA was determined by measuring the absorbance at 260 and 280 nm and calculating the ratio of 260/280 nm. The ratio was 1.8 which was within the range 1.7–1.9 required for DNA that was used for further molecular diagnostics. The quality and integrity of DNA samples were checked on 1% agarose gel after electrophoresis for 30 minutes.

Molecular diagnostics of Toxoplasma

B1 gene has a high specificity for detection of *T. gondii*, because it has been repeated 35 times in its genome, so it used as a target for amplification in PCR to detect the parasite in blood samples. The existence of *T. gondii* DNA in the maternal blood is likely to suggest a new, clinically significant, infection or apparent parasitaemia (tachyzoite).

A standard amplification plot with a mean C_t range: 25–28 was obtained. Electrophoretic analysis of the real-time PCR product showed an expected 98-bp band (Fig. 2). The threshold cycle value (C_t) was calculated to indicate the sum of the target gene at which the fluorescence exceeded the preset threshold, where the negative samples are shown as undetermined by the amplification plot. After 40 PCR cycles, the C_t values indicative of the quantity of the target gene were determined. The use of *B1* gene for *T. gondii* detection originated by [8] who combined PCR amplification with Southern blotting to detect a specific *B1* gene product since then, several variants of assays have been reported that have improved sensitivity or specificity.

In this study, RT-PCR was used for detection *B1* gene is considered as the best performing technique for diagnosis of congenital toxoplasmosis, compared with conventional PCR. These findings were stated by [21], which revealed that toxoplasmosis more prevalent among recurrent miscarriage cases in Babylon province. The present result also showed that the rate of positivity was 22.4 % in miscarriage women as shown in table 6.

According to the molecular results the amount of tachyzoites are five hundred within the infected samples and this agree with [30]. Current diagnosing of infection dependent on medical science detection it's may fail to detect specific anti-*Toxoplasma* IgG or IgM during the active phase of *T. gondii* infection, because these antibodies may not be produced until after several weeks of

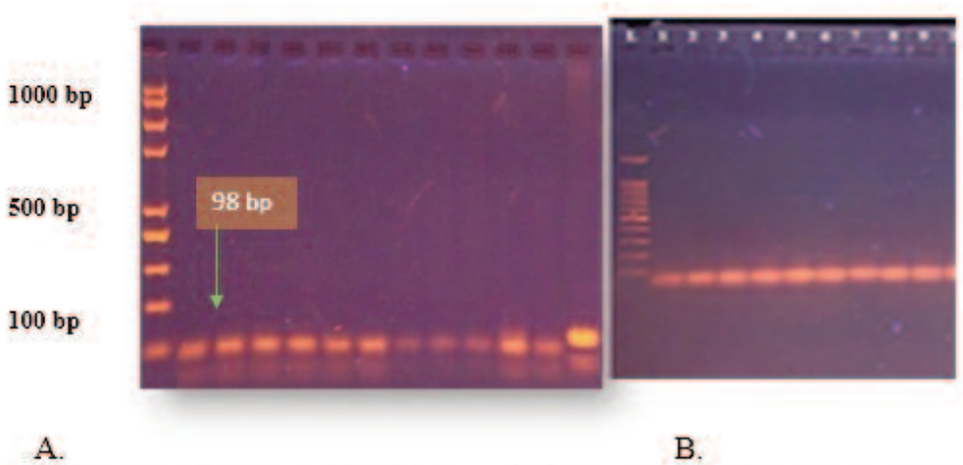

Figure 2. PCR product for detection *T. gondii* B1 gene after electrophoresis on 2% agarose gel. A: lane 1, DNA molecular marker with the positive results, B: lane 1–10, negative control

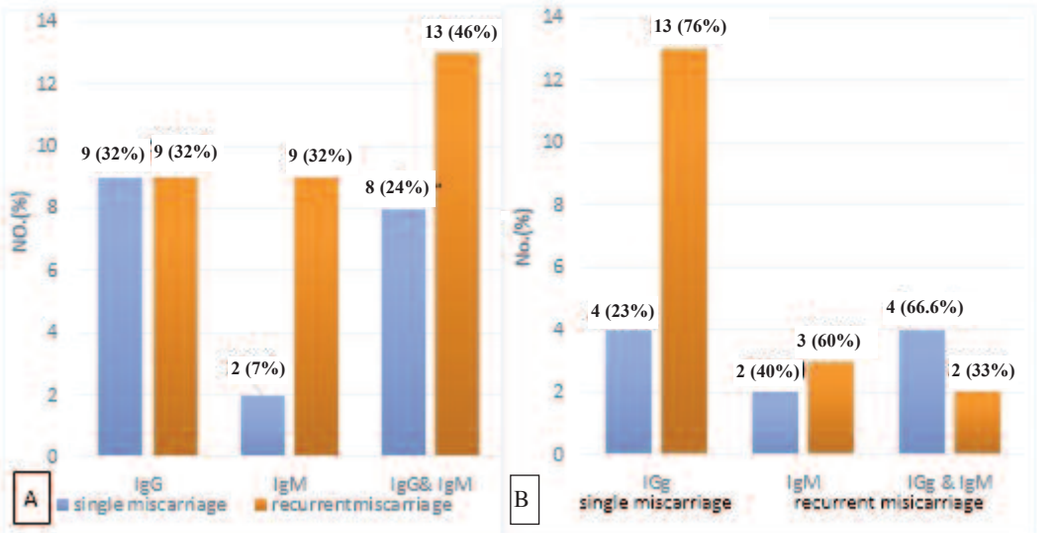

Figure 3. A: distribution of toxoplasmosis according ELISA test results in relation to antibodies type in TMW; B: distribution of toxoplasmosis according to RT-PCR results in relation to antibodies type in TMW

parasitemia. Therefore, in this study we used highly specific molecular as Real-Time PCR based on primers to amplify the *T. gondii B1* gene for detection of *T. gondii*.

In terms of the number of miscarriages and their distribution according to the diagnostic methods allowed in the study, recurrent miscarriage was comparatively recorded a high vital in compare with the single miscarriage cases 31(62%),18 (64%) respectively, as shown in figure 3.

This would suggest that in these miscarriage women, changes of IgG subclass levels leading to miscarriage. Pregnancies that ended in miscarriage showed a different pattern of IgG subclasses than those that continued to term. IgG 4 levels were significantly increased in continuing pregnancies, while miscarriage was associated with significantly reduced levels of IgG 4. This is presumably because levels of IL-4 are known to be reduced and levels of IFNγ are increased in these women [31]. Additionally, elevated levels of IFNγ are known to inhibit IgG 4 production.

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Received 07 July 2021
Accepted 30 September 2021