

## SCREENING TEST TO DETECT RECENT TOXOPLASMA GONDII INFECTIONS IN PREGNANT WOMEN

By

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### Abstract

The differentiation between acute and latent forms of *Toxoplasma gondii* (*T. gondii*) infection is still considered as a complicated issue. This study elucidated the establishment of a method that can detect most, if not all, of the recent infections with *T. gondii* through detecting both the immunological and genetic markers. The study included cases' group (G1): 30 pregnant women considered to be a high-risk group (with history of abnormal pregnancy outcomes), and the controls (G2) were 20 apparently healthy women cross matched in ages but with a history of normal pregnancy. The two groups were subjected to PCR and immunoblotting in the diagnosis of *T. gondii* infection compared to the routinely used assays (Rapid toxo IgM by immunochromatography & ELISA IgM).

The results showed that of 30 pregnant women (G1) 3(10%) were positive by Rapid Toxo-IgM, 5(16.67%) by ELISA-IgM, 9(30%) by immunoblotting and 6(20%) by PCR.

**Key words:** Pregnant women, PCR, ELISA, Immunoblotting, Rapid-Toxo-IgM.

### Introduction

Primary infections with *T. gondii* acquired during pregnancy are usually asymptomatic for the pregnant woman but can lead to serious neonatal complications (Linguissi, 2012). In countries such as the United States (Li *et al*, 2000) testing for antibodies to *Toxoplasma* in pregnancy is performed only in suspected cases. The first serum sample is usually taken at the first antenatal health care visit confirming pregnancy. The test to be used in such an antenatal screening should ideally be able to determine if the infection occurred after conception. Antenatal serological screening of *T. gondii* infection based on IgG and IgM detection is the mainstay in monitoring the risk for the congenital toxoplasmosis.

Among the serological tests, the indirect haemagglutination (IHA) proved to be more or less sensitive and more labor intensive (Malhotra *et al*, 1991). Indirect fluorescent antibody (IFA) test gave false-positive results with the rheumatoid factor, and the Sabin-Feldman dye test titres did not correlate with the severity of the toxoplasmosis illness (McCabe and Remington, 1983). Enzyme-linked immunosorbent assay (ELISA)

(both conventional and avidity) can be used for diagnosis and prognosis. ELISA has the sensitivity of about 88%, with a range of 83.3-98.6% and is 97% specific for clinching a diagnosis of toxoplasmosis. (Gamble *et al*, 2005). Routine serologic diagnosis of toxoplasmosis provides high sensitivity, but specificity varies depending on the test used, and false-positive IgM antibody test results have been reported by other investigators (Wilson *et al*, 1997). In addition, even true positive results must be interpreted with caution (Liesenfeld *et al*, 1997) because IgM antibodies may persist for 1 year after acute infection (Francis and Joynson, 1993).

Diagnosis of primary infection with *T. gondii* in early pregnancy can be improved by determination of anti-toxoplasma IgG avidity, which has the ability to discriminate between recent and prior infections (Pelloux *et al*, 1998). The high sensitivity, combined with the high specificity, offered by the PCR led us to investigate the presence of the *T. gondii* genome in maternal blood in cases of pregnancy where infections with this parasite were suspected. Previous studies confirmed that the PCR could actually detect *T. gondii* in blood specimens of women before

or during pregnancy (Hussein *et al.*, 2002). The clearance time for *Toxoplasma* DNA from the blood of patients with acute toxoplasmic lymphadenopathy was estimated to be during 5.5-13 weeks. Based on this, the presence of *Toxoplasma* DNA in the maternal blood most probably indicates a recent infection or an indicator of apparent parasitemia, which is likely to be clinically significant. However, a negative PCR result does not exclude recent infections because the sensitivity of the PCR, in which a single trophozoite can be detected in a clinical sample, has potential problems for some types of specimens, (Guy and Joynson, 1995) and because the exact kinetics of parasitemia in infected people are not well known. The short duration of parasitemia, or if the numbers of trophozoites circulating in peripheral blood are low, could lead to a sampling error that will produce false-negative results in such cases. The sensitivity of the PCR was found to be significantly higher for maternal infections that occurred between 17 and 21 weeks gestation ( $P < 0.02$ ) when the amniotic fluid was tested (Romand *et al.*, 2001) If the same applies to blood samples.

Serodiagnosis of recent infection in early pregnancy could be confirmed by PCR result in blood samples collected in the first half of pregnancy, due to the lack of sequential follow-up during pregnancy (Nimr *et al.*, 2004). Immunoblotting (IB) was recommended in congenital toxoplasmosis by three-IgM-band association at 75, 90, & 100 kDa called the IgM triplet increases the sensitivity to 95.8% when combined with prenatal and serological neonatal tests (L'Ollivier *et al.*, 2012). Sarkari *et al.* (2014) reported that anti *T. gondii* antibodies were detected in sera of 286/1,480 blood donors corresponding to an overall seroprevalence of 19.3% in this population by enzyme immunoassay. From these, 182 (12.3%) were seropositive only for IgG, 81 (5.47%) were seropositive only for IgM and 23 (1.6%) were positive for both IgG and IgM. PCR detected active

parasitemia in two (1.9%) of the IgM-positive subjects. these results highlighted that asymptomatic blood donors, especially those with active parasitemia, may constitute a significant risk of transmitting toxoplasmosis to susceptible recipients.

This is in contrary Zainodini *et al.* (2014) found that Out of 235 serum samples, 80 (34.04%) and 4 (1.71%) were positive regarding anti-*T. gondii* IgG and IgM antibodies, respectively, Real-Time PCR results showed that 14 out of 200 (6.97%) of blood donor had mRNA molecules of SAG1 gene. So evaluation of antibodies could not be reliable, because several donors negative for anti-*T. gondii* IgM antibodies had detectable SAG1 mRNA molecules. Hence, it seems that molecular diagnostic tests are essential to detect acute infections.

The present study aimed to evaluate the recent infections with *T. gondii*, by using PCR and immunoblotting as compared with the routinely used assays.

#### Material and Methods

This case control study was carried out from December 2010 and November 2013. Cases (G1) and controls (G2) were selected from patients attending at Obstetrics and Gynecology clinics, Al Hussein Hospital, Al- Azhar University. Two blood samples were collected from 50 eligible mothers selected based on their history of pregnancy. The cases' group included 30 pregnant women considered to be a high-risk group (with history of abnormal pregnancy outcomes, possible exposure to sources of *Toxoplasma*, previous screening revealing probable acquisition of *Toxoplasma gondii*). The controls were 20 apparently healthy women matched for age of the cases but with a history of normal pregnancy. All the cases and controls were subjected to the following.

- 1- Clinical evaluation all were clinical examined for toxoplasmosis manifestations.
- 2- Laboratory tests were done for all: Complete blood count (CBC) by using Coulter counter, Bilirubin (BIL) total and direct and C- reactive protein (CRP).

3- *Toxoplasma* specific IgM antibodies (*Toxo*-IgM) using commercial Rapid test: A lateral flow chromatography test where antibody complexes are allowed to move along a membrane strip chromatographically to be captured by the pre-coated recombinant specific antigens.

*Toxoplasma* specific IgM antibodies (*Toxo* IgM) by ELISA: Principle of the procedure: Competitive immunoassay. Incubation cycles: 1×30 minutes. They were measured as prescribed using Bioelisa *Toxo*-IgM immunocapture kits from (Bio-Kit, SA, Barcelona, Spain)

The blood samples (2-3cm each) were withdrawn aseptically by the vini-puncture into labeled test-tubes with cover (Remington and Desmonts, 1990). The separated non lipemic non haemolysed serum was stored frozen at (-20°C) until used for estimation of the *Toxoplasma* specific IgM titers, without addition of any preservative was used to avoid inhibition of the enzyme reaction

Interpretation of the test; Qualitative: Divided sample absorbance by the cut-off value: A ratio absorbance/cut-off >1.1 would be considered positive, a ratio absorbance / cut-off <0.9 would be considered negative, and a ratio absorbance/cut-off between 0.9 and 1.1 would be considered equivocal (Rigsby *et al*, 2004)

*Toxoplasma* specific B1 antigen detection in maternal samples by PCR: Blood samples were withdrawn and then preserved at -20°C. Samples were digested with Proteinase K in the supplied lysis solution. The lysate was then mixed with ethanol and loaded onto the purification column, where the DNA binds to the silica membrane. Impurities are effectively removed by washing the column with the prepared wash buffers. Genomic DNA is then eluted under low ionic strength conditions with the Elution Buffer.

DNA Extraction: Qiagen kit for extraction of DNA and Eppendorf centrifuge used during extraction of DNA

DNA amplification by PCR Analysis of products: Qiagen kit for cycling of DNA. Thirty µl of the PCR products were loaded on to 1.5% agarose gel and a unique band of 195 base pair (bp) was visualized using an ultraviolet transilluminator after ethidium bromide staining.

Negative controls for DNA extraction and for PCR assay, using respectively negative samples and water instead of DNA in the reaction mix, were included with all runs. Positive controls consisting of negative samples 'spiked' with 1 µg of *T. gondii* DNA (obtained from Tudor Bilharz institute) for DNA extraction and PCR reaction respectively, were also included.

Western blotting, Principle of test: *Toxoplasma gondii* antigens have been resolved by electrophoresis into bands and transferred by electro blotting on a nitrocellulose membrane (Western Blot procedure). The antigen bearing membrane has been cut into strips. Each strip is numbered and marked at the top of the upper side with a blue line. When doing a comparative immunological test, it is essential to use adjoining strips. The antigen bands at exactly the same level will allow easy interpretation of the test.

Western Blot-IgM: The strips are incubated with an alkaline phosphatase-anti human IgM conjugate. Specific-IgM anti-*T. gondii* antibodies, if present in the serum samples would then appear as violet colored bands on the strips.

Statistical analysis: Data was organized, tabulated and statistically analyzed using SPSS software statistical computer package version 13 (SPSS Inc, USA). For qualitative data number and distribution% were calculated. Chi (X<sup>2</sup>) square tested significance. P < 0.05 was for interpretation of tests of significance, considered significant and P < 0.001 was highly significant.

## Results

The results are shown in tables (1, 2 & 3).

Table 1: Comparison between G1 and G2 using Rapid Toxo-IgM, ELISA-IgM, Immunoblotting-IgM and PCR

Groups	Cases (G1)			Controls (G2)			Chi-Square
	Total (n=30)	Result	%	Total (n= 20)	%	X <sup>2</sup>	P-value
Rapid -IgM	27	Negative	90.00	20	100.00	7.216	0.065
	3	Positive	10.00	0	0.00		
ELISA-IgM	25	Negative	83.33	20	100.00	9.591	0.022*
	5	Positive	16.67	0	0.00		
Immunoblotting IgM	21	Negative	70.00	20	100.00	17.866	<0.001**
	9	Positive	30.00	0	0.00		
PCR	24	Negative	80.00	20	100.00	10.978	0.012*
	6	Positive	20.00	0	0.00		

P>0.05=non-significant, \* P< 0.05=significant, \*\* P< 0.001=high significant. *Toxo* Rapid IgM test showed non-significant increase in positive G1 comparison to G2. In ELISA-IgM, Immunoblotting IgM, and PCR showed significant increase in positive G1 in comparison to G2

Table 2: Immunoblotting with Rapid test, ELISA and PCR assay for IgM

Groups	Result	No.	Immunoblotting IgM				Chi-Square	
			30	Negative	%	Positive	%	X <sup>2</sup>
Rapid IgM	Negative	27	21	70.0	6	20.0	4.515	0.033*
	Positive	3	0	0.0	3	10.0		
Elisa IgM	Negative	25	21	70.0	4	13.33	8.670	0.003*
	Positive	5	0	0.0	5	16.67		
PCR	Negative	24	21	70.0	3	10.0	18.567	<0.00**
	Positive	6	0	0.0	6	20.0		

Rapid-IgM, ELISA-IgM and PCR with immunoblotting IgM were positive G1 in 3/9; 5/9 and 6/9 mothers with positive immunoblotting IgM results

Table 3; Results of the PCR assay with Rapid IgM test and ELISA IgM

Groups	Result	No.	PCR				Chi-Square	
			30	Negative	%	Positive	%	X <sup>2</sup>
Rapid Toxo-IgM	Negative	27	23	76.67	4	13.33	1.875	0.170
	Positive	3	1	3.33	2	6.67		
ELISA-IgM	Negative	25	23	76.67	2	6.67	11.082	0.001**
	Positive	5	1	3.33	4	13.33		

PCR with Rapid Toxo- IgM, and ELISA-IgM were positive G1 2/3 and 4/5 respectively and relation with ELISA *Toxo* IgM(P>0.05 non-significant).

### Discussion

In the present study, the 30 pregnant women were considered as high-risk group for acute infection by anti-*Toxoplasma* IgM immunoglobulins by the routinely used Rapid *Toxo*-IgM, ELISA-IgM and Immunoblotting-IgM and *Toxoplasma* specific B1 antigen was detected in maternal samples by PCR.

ELISA has been accepted as a sensitive and specific method for the routine laboratory diagnosis of both acute and chronic *Toxoplasma* infection in humans. An IgM-ELISA positive test alone or an increase of IgG concentration in two consecutive IgG-ELISA tests in a fortnight interval is regarded as an indicative of acute toxoplasmosis and the relevant parasitic stage i.e. tachyzoites. This is correct if compared with rapid card test; not goes with if compared with

Immunoblotting IgM or PCR; as The seroprevalence of *Toxoplasma* infection were 3/30(10%) positive by Rapid *Toxo*-IgM, 5/30 (16.67%) by ELISA IgM, 9/30 (30%) by immunoblotting and 6/30(20%) \by PCR with highest prevalence detected by Immunoblotting IgM. However, the presence of the persistent anti-*Toxoplasma* IgM antibodies, any positive IgM-ELISA could not definitely be interpreted as acute infection (Gross *et al*, 2004; Press *et al*, 2005). PCR for detecting specific *Toxoplasma* B1 antigen genetic markers of tachyzoites level in blood samples could overcome the above-mentioned problem. Moreover, the positive PCR were significantly higher than ELISA (6/30 positive by PCR vs. 5/30 by ELISA), also PCR detected 4/5 positive cases detected by ELISA, and PCR detected 2/25 nega-

tive cases by ELISA. False-positive ELISA-IgM antibody was reported (Hofgärtner *et al*, 1997; Wilson *et al*, 1997). Also, even true positive results must be interpreted with caution (Liesenfeld *et al*, 1997), as IgM antibodies might persist for one year after acute infection (Bobic *et al*, 1991; Francis *et al*, 1993). Sun *et al*. (2013) reported that the immunorecognition of three kinds of *T. gondii* strain and development specific antigens with the same set of sera. Results clearly showed that there were strikingly differences in the recognition of these antigens among the samples. The data further supported the conclusion that there is more genetic diversity among the *T. gondii* isolates in China (Zhou *et al*, 2009; Dubey *et al*, 2007) which argue for the necessity of the establishment of a method that can detect most, if not all, of the variant specific antibodies. so in this study Immunoblotting IgM detected more positive cases than other tests.

Generally, toxoplasmosis in women is a real problem not only in Egypt (Saleh *et al*, 2016), but worldwide as Saudi Arabia (Alanazi, 2013), Jordan (Morsy *et al*, 1978), Libya (Kassem and Morsy, 1991), Kuwait (Iqbal and Khalid, 2007), England (Eng *et al*, 1999), USA (Davis *et al*, 2015) and France (Armengol *et al*, 2017)

### Conclusion

Undoubtedly, congenital toxoplasmosis is a real health problem. The high sensitivity of the immunoblotting-IgM proved *T. gondii* in maternal blood of pregnant women where the infections were suspected.

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