

**DETECTION TOXOPLASMA GONDII BY REAL-TIME PCR IN ABORTIVE AND
PREGNANT WOMEN IN AL-MUTHANNA PROVINCE**

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ABSTRACT

The protozoan *Toxoplasma gondii* is one of the most common infectious pathogenic parasites and can cause severe medical complications in infants and immune compromised individuals. We report here the development of a real-time PCR-based assay for the detection of *T. gondii*. Primary maternal infection with toxoplasmosis during gestation and its transmission to the fetus continue to be the cause of tragic yet preventable disease in offspring. This study was aimed to investigate the utility of nested PCR (RT-PCR) technique for detection recent infection with *Toxoplasma gondii* in blood of pregnant and abortive women. One hundred fifty women were included in this study with a history of single or repeated abortion and thirty women with normal pregnancy were used as a control. Blood samples were tested for specific anti-*Toxoplasma* IgM and IgG antibodies by an enzyme-linked immunosorbent assay (ELISA) and detection of B1 gene of *T. gondii* by RT-PCR. The results indicated that 59% of abortive women were exposed positive for anti- *Toxoplasma* antibodies, 12% of them had IgM, 42% had IgG, and 5 % had both IgM and IgG, and 41% had no antibodies. Subsequently, RT- PCR analysis was used to detecting *T. gondii* DNA in blood of abortive women. It was found that 17.7% of abortive women exposed positive result for B1 gene of *T. gondii*, those abortive women involved 41.667% of them with IgM, 23.81%with IgG, and 40% with both IgM and IgG, and 0% of them had none anti-*Toxoplasma* antibodies.

KEYWORDS: *Toxoplasma Gondii*, RT-PCR, B1 Gene

INTRUDACTION

Toxoplasmosis is a common parasitic disease caused by the protozoan parasite *Toxoplasma gondii*. Its prevalence in humans varies from region to region depending on ecological and cultural factors (Dupouy-Camet et al., 1993). Current diagnosis of toxoplasmosis relies either on serological detection of specific anti-*Toxoplasma* immunoglobulin, on culture of amniotic fluid or fetal blood, or on other nonspecific indicators of infection (James et al., 1996; Remington et al., 1995). In general, *T. gondii*

infections are asymptomatic and self-limiting especially among healthy immune competent hosts; however the infection may cause severe complications in pregnant women and immune compromised patients (Espinoza 2005; Holland et al., 1996). Fetal toxoplasmosis, particularly in early pregnancy can cause miscarriage, stillbirth, and birth defect. Infected babies may not develop any disease, or they may experience serious damage to the brain and eyes, to degree depending on the gestational age (Montoya and Remington, 2008). The laboratory diagnosis of toxoplasmosis can be performed in several ways (Fleck and Kwantes, 1980) including histological examination, the isolation of the parasite after inoculation, and several serological methods. None of these methods offer a good and reliable result for toxoplasmosis, particularly in the fetus and immune suppressed patients (Savva et al., 1990). The polymerase chain reaction (PCR) has been used as an alternative to serology by amplification of *Toxoplasma* DNA sequences present in various clinical samples, such as amniotic fluid (Jenum et al., 1998), cerebrospinal fluid (Cingolani et al., 1996), tissues (Held et al., 2000), aqueous humor (Mahalakshmi et al., 2006), and human blood (Kompalic-Cristo et al., 2007). Among these techniques, nested PCR followed by hybridization of PCR products has been the most sensitive method. However, the major disadvantage of these methods is that they are quite time-consuming and do not provide quantitative data. The recent advent of a real-time quantitative PCR technique has proven useful in various applications, including pathogen detection, gene expression and regulation, and allelic discrimination (Das et al., 2000; Fujii et al., 2000; Takeuchi et al., 1999). Real-time PCR utilizes the 5' nuclease activity of Taq DNA polymerase (Holland et al., 1991) to cleave a non extendible, fluorescence- labeled hybridization probe during the extension phase of PCR. The fluorescence of the intact probe is quenched by a second fluorescent dye, usually 6-carboxy-tetramethyl- rhodamine (TAMRA). This study, we describe the development of a real-time quantitative PCR for the detection of *T. gondii*. The use of this methodology will facilitate the diagnosis of *T. gondii* in clinical laboratories.

MATERIALS AND METHODS

Study groups

The cases were collected during the period from 1st January to the end of March 2013 from in the AL-Samawa feminine and Children teaching hospital in AL-Muthanna province. They were included 100 women, who had spontaneous abortion .In addition, 50 healthy women with a history of a normal pregnancy were attended to the outpatient clinics for routine gynecologic checking was considered as control. Relevant information about the

cases and the possible risk factors were recorded for each woman. Any woman undergoing therapy against the disease was excluded from the study.

Serological test

The sera of all cases were tested for the presence of specific IgM and IgG anti-Toxoplasma antibodies via ELISA kits (Biokit Diagnostics Company, Spain) according to the manufacture's instructions.

Isolation of genomic DNA from whole blood

DNA was extracted from the whole blood samples of the study groups using a commercial purification system (Reagent Genomic DNA extraction kit (Geneaid. USA)) following the manufacture's instruction for DNA purification from blood. Purified DNA molecules were resuspended in 100 µl of DNA rehydration solution (TE buffer) (10 mM Tris-HCL, 1mM EDTA, pH=8) and stored at -80°C, after estimation of DNA concentration and purity, The extracted genomic DNA from whole blood samples was checked by using Nanodrop spectrophotometer (THERMO.USA), that check and measurement the purity of DNA through reading the absorbance in at (260 /280 nm).

Detection of T. gondii B1 gene by real-time quantitative PCR

The forward primer (TOXO-F), reverse primer (TOXO-R), and Taq Man probe for real-time PCR amplification were designed with the Primer Express software (PE Applied Bio system) to specifically amplify the T. gondii B1 gene. The target DNA for real-time PCR amplification was the published sequence of the 35-fold repetitive B1 gene of the T. gondii RH strain (Burg et al., 1989). Briefly, template DNA was added to a reaction mixture containing 25 µl of 23 PCR universal master mix, 5 µl of the forward primer TOXO-F (5 mM, 59-TCCCCCTCTGCTGGCGAAAAGT-39), 5µl of the reverse primer TOXO-R (5 mM, 59-AGCGTTCGTGGTCAACTATCGATTG-39), and 5 µl of TaqMan probe (2 mM, 6FAM-TCTGTGCAACTTTG GTGTATTCGCAG-TAMRA) in a final volume of 50 µl. The PCRs were performed with the Gen Amp 5700 Sequence Detection System (PE Applied Bio system). After initial activation of AmpliTaq Gold DNA polymerase at 95°C for 10 min, 40 PCR cycles of 95°C for 15 s and 60°C for 1 min were performed. The cycle threshold value (CT), indicative of the quantity of target gene at which the fluorescence exceeds a preset threshold, was determined. This threshold was defined as 20 times the standard deviation of the baseline fluorescent signal, i.e., the normalized fluorescent signal of the first few PCR cycles. After reaching the threshold, the sample was considered positive.

Statistical analysis

Data were analyzed with chi-square and P value < 0.05 was considered statistically significant.

Results

Figure (1) shows that Only 12% of the 150 abortive women tested positive for IgM antibodies compared with 0% and 0% (none) in the a normal pregnancy and control group respectively. While for IgG antibodies 42% of abortive women tested positive compared with 3% and 0% positive IgG antibodies in a normal pregnancy and in the control group respectively. Both IgM and IgG seropositive antibodies appeared in 5% in abortive women while it was 0% in a normal pregnancy and 0% in the control group. Negative results appeared in 41%, 88% and 100% in abortive women, a normal pregnancy and control group respectively. These differences were statistically significant (P< 0.05).

Figure (2) shows that By RT-PCR technique, the study revealed that out of 96 suspected cases, only 17.7% showed positive toxoplasmosis, A typical amplification plot (change in fluorescent signal versus cycle numbers) with a CT of 25.1 was obtained. The cycle threshold value (CT), indicative of the quantity of target gene at which the fluorescence exceeds a preset threshold was determined, where the negative samples show as undetermined by amplification plot show in figure (3). Besides the serological diagnosis of T.gondii real time PCR (RT-PCR) technique was used to confirm the infection with T.gondii by detection of T. gondii DNA in the blood of abortive women. Toxoplasma gondii DNA was successfully extracted and analyzed by RT-PCR. Abortive women 17% who recorded positive results in RT-PCR analysis were distributed on the patterns of the anti-Toxoplasma antibodies , it was found that 5(41.7%) of them appeared with IgM⁺ pattern and 10(23.8%) with the both patterns of IgG⁺ and no anti-Toxoplasma antibodies. In addition, 2(26.31%) of a positive RT-PCR results revealed both (IgM⁺ and IgG⁺) antibodies. Whereas, the negative RT-PCR analysis was significantly (P < 0.05) formed 82.29% in abortive women when compared with control group (100%) show in figure (4).

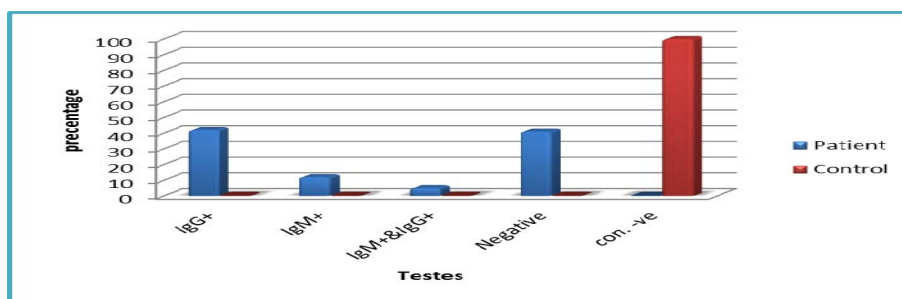


Figure (1): Seroprevalence of anti-Toxoplasma antibodies by ELISA in the subjects of the study.

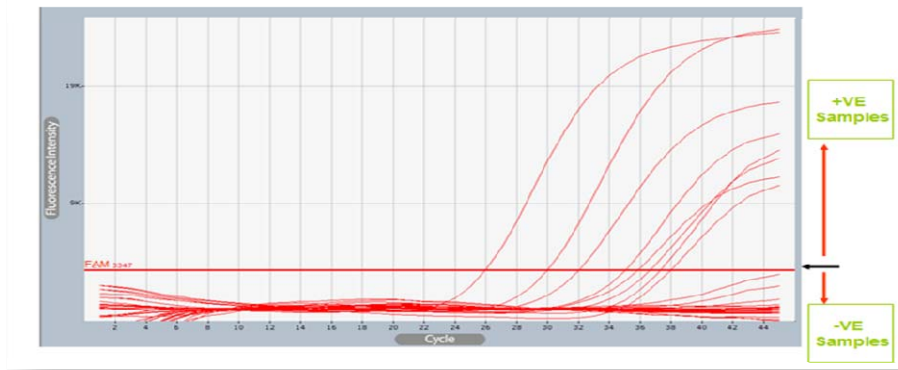


Figure (2): Real-Time PCR amplification plot shown the positive and negative *Toxoplasma gondii*.

Absolute Quantification : 2013_4_25_10_25_27_406 HAWRAA TOXOPLASMA.ex3

File View Setup Help

Flu. Graph Well vs Ct Plate Info Report Form

	Well	Type	Flu. Dye	Quencher	Ct	Start Baseline	End Baseline
1	A1	Sample	FAM	TAMRA	25.1	Auto	Auto
2	A2	Sample	FAM	TAMRA	Undetermin	Auto	Auto
3	A3	Sample	FAM	TAMRA	Undetermin	Auto	Auto
4	A4	Sample	FAM	TAMRA	Undetermin	Auto	Auto
5	A5	Sample	FAM	TAMRA	38.14	Auto	Auto
6	A6	Sample	FAM	TAMRA	Undetermin	Auto	Auto
7	A7	Sample	FAM	TAMRA	Undetermin	Auto	Auto
8	A8	Sample	FAM	TAMRA	Undetermin	Auto	Auto
9	A9	Sample	FAM	TAMRA	Undetermin	Auto	Auto
10	A10	Sample	FAM	TAMRA	Undetermin	Auto	Auto
11	A11	Sample	FAM	TAMRA	Undetermin	Auto	Auto
12	A12	Sample	FAM	TAMRA	35.11	Auto	Auto
13	B1	Sample	FAM	TAMRA	38.07	Auto	Auto
14	B2	Sample	FAM	TAMRA	Undetermin	Auto	Auto
15	B3	Sample	FAM	TAMRA	Undetermin	Auto	Auto
16	B4	Sample	FAM	TAMRA	Undetermin	Auto	Auto
17	B5	Sample	FAM	TAMRA	Undetermin	Auto	Auto
18	B6	Sample	FAM	TAMRA	Undetermin	Auto	Auto
19	B7	Sample	FAM	TAMRA	Undetermin	Auto	Auto
20	B8	Sample	FAM	TAMRA	Undetermin	Auto	Auto
21	B9	Sample	FAM	TAMRA	Undetermin	Auto	Auto
22	B10	Sample	FAM	TAMRA	Undetermin	Auto	Auto
23	B11	Sample	FAM	TAMRA	Undetermin	Auto	Auto
24	B12	Sample	FAM	TAMRA	Undetermin	Auto	Auto
25	C1	Sample	FAM	TAMRA	Undetermin	Auto	Auto
26	C2	Sample	FAM	TAMRA	Undetermin	Auto	Auto
27	C3	Sample	FAM	TAMRA	Undetermin	Auto	Auto
28	C4	Sample	FAM	TAMRA	Undetermin	Auto	Auto
29	C5	Sample	FAM	TAMRA	Undetermin	Auto	Auto
30	C6	Sample	FAM	TAMRA	Undetermin	Auto	Auto
31	C7	Sample	FAM	TAMRA	Undetermin	Auto	Auto
32	C8	Sample	FAM	TAMRA	Undetermin	Auto	Auto
33	C9	Sample	FAM	TAMRA	Undetermin	Auto	Auto

+VE Samples

Figure (3): Real-Time PCR data analysis shown the threshold cycle number (CT) for positive *Toxoplasma gondii*, where the negative samples show as undetermined by amplification plot.

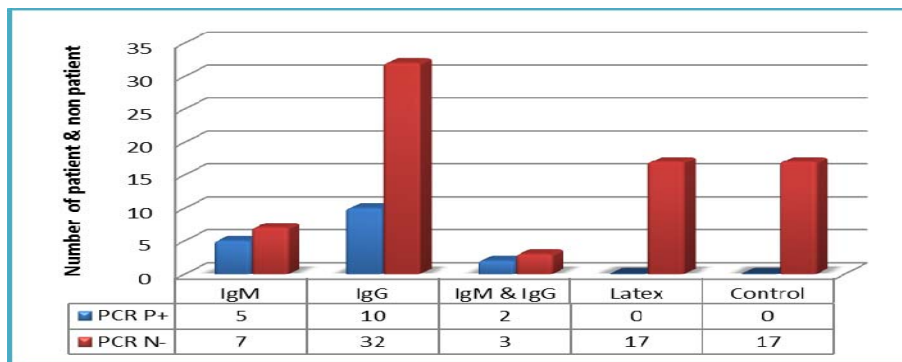


Figure (4): RT- PCR analysis distributed according to anti-Toxoplasma antibodies in abortive women and control group.

Discussion

The most likely sources of human toxoplasmosis are ingestion of lightly cooked meat containing live *T. gondii* tissues cysts, ingestion of vegetable or fruits contaminated with oocysts derived from cat faeces that may be encountered in gardens. Toxoplasmosis is also recognized to be a water-borne zoonosis (Dubey, 2004; Qublan et al., 2002). PCR has been used in an effort to overcome serious deficiencies of other tests for the detection of *Toxoplasma* DNA in infected tissues, particularly in cases of immune suppressed and immune compromised individuals (Savva et al., 1990). The diagnostic value of PCR for the detection of *T. gondii* in blood samples has been evaluated from both immune-competent and compromised patients (Bou et al., 1999; Ho-Yen et al., 1992; Kompalic-Cristo et al., 2007). 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* (Ellis, 1998), including the B1 gene repetitive sequence, the P30 (SAG1) gene, and ribosomal DNA. The use of the B1 gene for *T. gondii* detection originated with Burg et al. (1989), who combined PCR amplification with Southern blotting to detect a specific B1 gene product. Since then, several variations of assays have been reported that have improved sensitivity or specificity. For example, Pujol-Rique et al. (1999) designed a one-tube heminested PCR method with a sensitivity equivalent to 0.1 parasite (Pujol-Rique et al., 1999). Although serological testing has been one of the major diagnostic 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 due to the fact that the titers of specific anti-Toxoplasma antibodies may fail to rise in this type of patient. Indeed detection of *T.*

gondii DNA using RT-PCR minimizes the problems faced when using sero diagnostic assays and facilitates diagnosis in difficult cases. Therefore, the negative results obtained by both PCR and ELISA rule out an infection in abortive women. The fact that could explain the proportion of 58.333% of abortive women that revealed negative PCR results and positive IgM is the probability to find patients with residual IgM detected during prolonged periods of time. Also false positive IgM tests results have been reported previously (Emna et al., 2006). Toxoplasma DNA was found in 23.81% abortive women with positive IgG indicates chronic infection since it is known that patients with latent toxoplasmosis present intermittent parasitemia with low parasite burden. Toxoplasma DNA was detected in 76.19% seronegative abortive 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. 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 infection, 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 (Bastien, 2002). The real-time PCR-based method described in this study provides a rapid, sensitive, and quantitative way of detecting *T. gondii* in clinical specimens.

Conclusion

We have developed a rapid, sensitive, and quantitative real-time PCR for detection of *T. gondii*. The advantages of this technique for the diagnosis of toxoplasmosis in a clinical laboratory are discussed.

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