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MOLECULAR DETECTION OF *TOXOPLASMA GONDII* IN WOMEN AT WASIT PROVINCE, IRAQ

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Abstract

The *Toxoplasma gondii is* one of the most common zoonotic infectious pathogenic parasites and can be infected the human and animals. Regarding to transmission of disease by feline. The current study aimed to diagnose *T. gondii* by highly specific molecular as Real-Time PCR based TaqMan probe and primers to amplify the B1 gene of *Toxoplasma gondii*. The present study included 200 blood samples of the pregnant and non- pregnant women attending to the Al- kut and Al- Shaheed Fairose Hospitals during October 2023-December 2023 to identify the prevalence of the parasitic disease of *Toxoplasma gondii* among females .

The present results showed that the B1 gene for detection *T. gondii* were reported in 11 (11.0%) of pregnant women and 10 (10.0%) of non-pregnant women. The higher infection 8/54 (12.9%) was appeared in age group (20-29) years old of the pregnant women and in the rural areas 9/42(17.6%). The aborted women was reported the highest infection of *T. gondii* 9/29 (23.7%).
.**Keywords :** Women, Abortion , *T. gondii* , Real-Time PCR

Introduction:

Toxoplasmosis is parasitic disease caused by *Toxoplasma gondii*, one of the most prevalent causes of abortion and congenital deformity in women (Paquet *et al*., 2013). It is a zoonotic disease, one of the first parasitic cells Obligate intracellular which belongs to the Apicomplexa division, causes various clinical symptoms including encephalitis, placenta and retinitis, and loss of sight in children with (Abdallah *et al*., 2019;Tilahun *et al*., 2018). About one third of the world's population is considered to be infected with infection, and aromatization is mostly transmitted to humans by consuming toxoplasma-contaminated food and water as well as raw meat, especially lamb with a tissue bag for the parasite; In rare cases, it can also be transmitted by contaminated eggs or raw milk (Dubey *et al*., 2020). The only final host of this parasite is the domestic and land cats in which the parasite Oocysts are present in the parasite and play an active role in the transmission of the injury and the occurrence of sexual reproduction (Knoll *et al*., 2019). Since domestic cats are one of the most human companions, as a result, frequent contact with them increases the risk of *T.gondii* infection (Salman *et al*., 2018). The disease is present in two forms depending on the occurrence of infections, the first congenital toxoplasmosis, which is transmitted from mother to fetus through sill age and may lead to abortion in pregnant women, stillbirth or severe mental retardation (Ghasemi *et al*., 2016). The second form, acquired toxoplasmosis, generally occurs by consuming undercooked meat containing textile bags or by water and food contaminated with egg bags found in cat stool (Rogerio *et al*., 2011). The diagnosis of the *Toxoplasma* parasite is done by investigating antibodies whether it be for acute IgM or chronic IgG infusion as well as genetic test PCR Polymerase chain reaction which is of high diagnostic accuracy (Aldabagh *et al*., 2018; Young *et al*., 2018) .

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Materials and Methods:

Samples collection:

A total of 200 blood samples were collected from pregnant and non- pregnant women attending to the Al- kut and Al- Shaheed fairose Hospital during October, 2023 to December 2023. A questionnaire form was applied by each female which included: Pregnancy, abortion, age and residence. The collected blood samples kept in clean and sterilized test tubes with anticoagulant for DNA extraction and transported in to the laboratory. Non-clotted blood samples were stored in - 20°C freeze until used for genomic DNA extraction.

Real Time PCR Primers

The primers and probe that used for direct detection of *Toxoplasma gondii* by Real-Time PCR were designed by (Mei-Hui Lin *et al*., 2000) and these provided by (Macrogen, Korea) as following tables:

Table (1) : Primers and Probe for *Toxoplasma gondii*

Primer	Sequence (5'-3')		Product Size
B1- primer	F	TCCCCTCTGCTGGCGAAAAGT	113 bp
	R	AGCGTTCGTGGTCAACTATCGATTG	
B1- probe	FAM-TCTGTGCAACTTTGGTGTATTCGCAG-TAMRA		

Genomic DNA Extraction

Genomic DNA from blood samples were extracted by using gSYAN DNA kit extraction kit (Frozen Blood) Geneaid. USA, and done according to company instructions as following steps:

1. A 200µl of frozen blood was transferred to sterile 1.5ml microcentrifuge tube, and then added 30µl of proteinase K and mixed by vortex. And incubated at 60℃ for 5 minutes.
2. After that, 200µl of lysis buffer GSB was added to each tube and mixed by vortex vigorously, and then all tubes were incubated at 70℃ for 10 minutes, and inverted every 3 minutes through incubation periods.
3. 200µl absolute ethanol were added to lysate and immediately mixed by shaking vigorously.
4. DNA filter column was placed in a 2 ml collection tube and transferred all of the mixture (including any precipitate) to column. Then centrifuged at 10000rpm for 5 minutes. And the 2 ml collection tube containing the flow.through were discarded and placed the column in a new 2 ml collection tube.
5. 400µl W1 buffer were added to the DNA filter column, then centrifuge at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube.
6. 600µl Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow.through was discarded and placed the column back in the 2 ml collection tube.
7. All the tubes were centrifuged again for 3 minutes at 10000 rpm to dry the column matrix.

8. The dried DNA filter column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of pre.heated elution buffer were added to the center of the column matrix.
9. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

Real-Time PCR :

Real-Time PCR technique was performed for in detection of *Toxoplasma gondii* on B1 gene amplification. technique was carried out according to method described by (Gault *et al*., 2001) as following steps:

Real-Time PCR master mix preparation:

qPCR master mix was prepared by using (**RealMOD^{TM} probe 2X qPCR mix**) and this master mix done according to company instructions as following table (2):

PCR Master mix	Volume
DNA template	5µL
B1 gene Forward primer (10pmol)	1µL
B1 gene Reverse primer (10pmol)	1µL
B1 gene probe (20pmol)	1µL
qPCR master mix	10 µL
Nuclease free water	2 µL
Total volume	20µL

After that, these PCR master mix component that mentioned in table above transferred into Exispin vortex centrifuge at 3000 rpm for 3 minutes. Then placed in Real-time PCR Thermocycler (BioRad , USA).

Real-Time PCR Thermocycler conditions:

Real-Time PCR thermocycler conditions was set according to primer annealing temperature and qPCR TaqMan kit instructions by Bio-Rad Real-Time PCR thermocycler system as in the following table (3):

Table (3): PCR thermocycler conditions

Step	Condition	Cycle
Pre-Denaturation	95 °C 5 min	1
Denaturation	95 °C 20 sec	40
Annealing/Extension	60 °C 30 sec	
Detection (Scan)		

Real-Time PCR Data analysis:

qPCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive viral DNA copy numberin Real-Time PCR cycle number.

Results:

The results of polymerase chain reaction for the detection of *B1 gene* of *T. gondii*

The frequency distribution of pregnant women and non-pregnant women according to the results of PCR for detection B1 gene of *T. gondii* was shown in table (4). The present result showed the B1 gene for detection *T. gondii* were reported in 11 (11.0%) of pregnant women and the B1 gene for detection *T. gondii* showed in 10 (10.0%) of non-pregnant women.

Table (4): Prevalence *T. gondii* infection according to PCR findings in studied groups

Characteristics	Pregnant women n = 100	Non-pregnant women n = 100	P value
B1 gene of PCR			
Positive, *n* (%)	11 (11.0%)	10 (10.0%)	0.818 ¥ NS
Negative, n (%)	89 (89.0%)	90 (90.0%)	

¥: Chi-square test; NS: non-significant at P <0.001.

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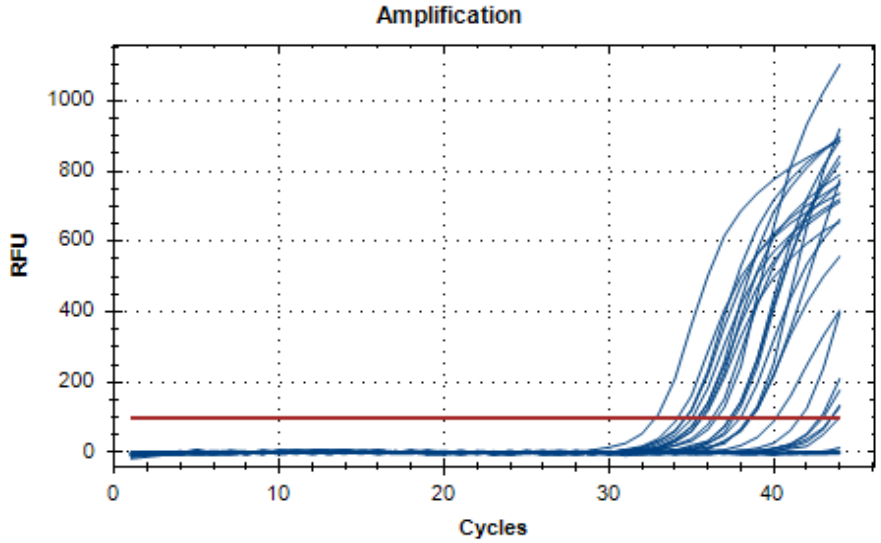

Figure 1: qPCR plot showing amplification of the B1 gene to detect Toxoplasma gondii. The plot shows cycles on the x-axis and relative fluorescence units (RFU) on the y-axis. RFU is a measurement of fluorescent light emitted and is proportional to the amount of PCR product in the reaction. A qPCR experiment has a threshold line set above the background fluorescence. As the PCR cycles progress and more product is amplified, the fluorescence intensity increases. The positive sample containing *T. gondii* DNA which appeared fluorescence intensity crosses the threshold line within 31-40 cycles (Ct). The negative samples that did not contain *T. gondii* DNA did not cross the threshold line within 40 cycles.

Frequency distribution of patient groups according to age groups

With regard to polymerase chain reaction results, the frequency distribution of *T. gondii* positive results in pregnant women by age group included 1 cases in the less than 20 years age group, 8 cases in the 20-29 years age group and only 2 cases in the more than 30 years age group. Whereas the frequency distribution of *T. gondii* positive results in non-pregnant women were 5 cases in the 20-29 years age group and 5 cases in the more than 30 years age group, while no positive cases in the less than 20 years age group, and the difference was non-significant at (P<0.05).

Table(5): Number and percentage of pregnant and non-pregnant women according to age groups by PCR results

Characteristic	*Pregnant women* +/-	*Non-pregnant* +/-	P value ¥
Age (years)			
< 20, *n* (%)	1/15 (6.2%)	0/10 (0)	0.420
20-29, *n* (%)	8/54 (12.9%)	5/48 (9.4 %)	0.558
≥ 30, *n* (%)	2/20 (9.1%)	5/32 (13.5%)	0.611
P value ¥	0.712	0.441	

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Calculated X2	0.680	1.637	

n: number of cases; ¥: Chi-square test; *****: significant at *P* <0.05

Frequency distribution of patient groups according to residency

Polymerase chain reaction results, the frequency distribution of *T. gondii* positive results in pregnant women according to residence included 2 cases from urban areas and 9 cases from rural areas, while in pregnant women with *T. gondii* negative results according to residence, 47 cases from urban areas and 42 cases from rural areas and the differences was significant (P= 0.030). While the frequency distribution of *T. gondii* positive results in non-pregnant women according to residence was 4 cases from urban areas and 6 cases from rural areas, while in the non-pregnant women with *T. gondii* negative results according to residence, 61 cases from urban areas and 21 cases from rural areas but the differences was non-significant (P= 0.081).

Table (6): Number and percentage of pregnant and non-pregnant women according to the residency by PCR

Characteristic	**Pregnant women** +/-	**Non-pregnant** +/-	**P value** ¥
Residency			
Urban, n (%)	2/47 (4.1%)	4/61 (6.2 %)	0.427
Rural, n (%)	9/42(17.6%)	6/29 (17.1%)	0.952
P value ¥	0.030*	0.081	
Calculated X^2	4.697	3.053	

n: number of cases; ¥: Chi-square test; *****: significant at P <0.05

Frequency distribution of pregnant women and non-pregnant women according to abortion

Regarding polymerase chain reaction results, the frequency distribution of *T. gondii* positive results in pregnant women according to abortion included 9 cases with positive abortion and 2 cases with negative abortion, while in pregnant women with *T. gondii* negative results according to abortion, 29 cases with positive abortion and 60 cases with negative abortion and the differences was significant (P= 0.002). While the frequency distribution of *T. gondii* positive results in non-pregnant women according to abortion was 5 cases with positive abortion and 5 cases with negative abortion, while in the non-pregnant women with *T. gondii* negative results according to abortion, 22 cases with positive abortion and 68 cases with negative abortion and the differences was non-significant (P= 0.084).

Table (7): Number and percentage of pregnant and non-pregnant women according to abortion by polymerase chain reaction results

Characteristic	**Pregnant women** +/-	**Non-pregnant** +/-	**P value** ¥
Abortion			

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Positive, *n* (%)	9/29 (23.7%)	5/22 (18.5 %)	0.618
Negative, n (%)	2/60 (3.2%)	5/68 (6.8%)	0.344
P value ¥	0.002*	0.084	
Calculated X^2	10.072	2.982	

n: number of cases; ¥: Chi-square test; *****: significant at P <0.05

Discussion:

Molecular methods based on polymerase chain reaction (PCR) are simple, sensitive, reproducible and can be applied to all clinical samples (Bell and Ranford, 2002; Contini *et al*., 2005). The first protocol for molecular detection of *T. gondii*, for conventional PCR targeting B1gene, was developed in 1989 and has since been modified and optimized in many laboratories (Reischl *et al*., 2003; Switaj *et al*., 2005). The B1 gene, although of unknown function, is widely exploited in a number of diagnostic and epidemiological studies because of its specificity and sensitivity. There are also some studies in which the detection of *T. gondii* parasites was based on amplification of ITS-1 and 18S rDNA fragments, whose sensitivity was similar to the B1 molecular detection and Genotyping of *T. gondii* from clinical samples 105 gene (Hurtado *et al*,. 2001; Caldearo *et al*., 2006).

The current study showed that the rate of infection in women according to the PCR test amounted 11 % in pregnant women and 10% in non-pregnant women. This may be attributed to the presence of the tachyzoite in blood during the acute phase of the disease and leaving to the tissues during the chronic phase. Probably the reason for the fact that a positive result in the two chronic cases that these two cases are with sub-acute phase and a few number of tachyzoite still exists in the blood (Naot and Remington, 1980).

There may be other studies referred to the highly sensitivity of PCR test when performed to detect *T. gondii* in amniotic fluid, placenta and umblical cord blood (Chabber *et al*., 2004). Previous studies have documented that PCR can detect the parasite DNA in blood samples of women before or during pregnancy (Chabber et al., 2004). The presence of *Toxoplasma* DNA in maternal blood probably indicates a recent infection or apparent parasitemia, which is likely to be clinically significant (Slawska *et al*., 2005).

PCR is the only method that can detect low levels of the *T. gondii* organism and even destroyed parasites (Savva *et al*., 1990). The results presented by (Burg *et al*., 1989) showed that a single *T. gondii* parasite could be detected by PCR. Another explanation for the high rate of positive test results by PCR is that the amplification of B1 could represent samples containing the parasite DNA but no viable pathogens, as the PCR test does not rely on live parasites to show a positive result (Wastling *et al*., 1993). This result was nearly similar to the result of 17.65% (Al-Kalaby, 2008) of tested samples from Iraqi women was positive by PCR technique using B1 gene, while disagreed with the results of (Al-Addlan, 2007; Okay *et al*,. 2009) who reported that who recorded that 83.3% and 63.49% respectively.

The differences among percentages that, recorded in the current study and other studies, may be attributed to the different origins of used samples (blood, amniotic fluid, placenta tissue, etc.), and this interpretation was stated by (Marie-Francoise *et al*., 1999; Al-Kalaby,

2008) when they recorded a significant differences among different used samples, or due to immunocompetent or immunocompromised status of patients (Luft and Remington 1992; Israelski and Remington 1993).

Conclusions:

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.

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