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Epidemiological Study on the Evaluation of Molecular and Immunological assay for the Detection of *Toxoplasma gondii* in Women's Abortion

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ABSTRACT

Toxoplasmosis, caused by *Toxoplasma gondii*, is one of the most prevalent parasitic infections in humans. At its primary stages, this infection is usually asymptomatic in a pregnant woman; however, it has the potential to cause significant harm to the fetus, including miscarriage. The current study was investigated the utility of the PCR to confirm the etiology of the abortion. Therefore, a prospective study was conducted on 94 aborted women hospitalized at Al-Diwaniyah Maternity and Pediatric Teaching Hospital, Iraq. To detect toxoplasmosis, a new internal primer for the nested PCR protocol was introduced. Of the 94 aborted women, 30 samples (31.9%) were positive by the nPCR and qPCR using the G529 repeat gene and B1 gene primers, respectively. The findings indicated that three women carried the parasite in their placentas, and at the same time, they did not carry antibodies in their blood. In conclusion, women should be aware of the risk of toxoplasmosis and the importance of preventing measures. In addition, PCR should be performed in the case of abortion to enhance sensitivity even if the serological test result is negative.

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Introduction

Abortion is a serious problem that women may face during pregnancy leading to psychological pressures and medical expenses (1). *Toxoplasma gondii* (*T. gondii*), an obligatory intracellular parasite of the genus Sporozoa, is one of the causes of miscarriage. It causes a foodborne or waterborne parasitic protozoan infection (2). *Toxoplasma* is a parasite that is spreading in hot and humid countries and can infect a wide variety of vertebrate hosts, cats, and members of the Felidae family which are the definitive hosts (3).

The development of an accurate, sensitive, and rapid method for the detection and identification of *T. gondii* is important for both the diagnosis and treatment of humans and animals (4,5). In recent years, efforts have been made to improve the ability to diagnose parasite infections in pregnant women and congenital infections in the fetus and newborn (6-8).

In Iraq, the abortion rate due to toxoplasmosis is unknown, and most of the conducted studies have relied on serological tests. Therefore, the present study was an attempt at investigating the utility of the Enzyme-linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) to show the presence of *T. gondii* in aborted women and their placentas.

Materials and methods

Study area

The current prospective study was conducted on a total of 94 aborted women hospitalized at the Al-Diwaniyah Maternity and Pediatric Teaching Hospital in Iraq. The study was lasted nine months from December 2020 to August 2021.

Participates and sampling

The participants within the age range of 18 to 40 years and with no history of internal diseases, trauma and or any other effective factors in abortion were included in the current study. Demographic

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information, including factors influencing the epidemiology of *Toxoplasma*, such as maternal age, trimester time abortion, maternal occupation, place of residence, level of education, and contact with animals, were collected from the participants. After recording the epidemiologic characteristics, a 5 mL blood sample was collected from each participant in an EDTA-containing tube. All placentas and blood samples were transferred immediately to the laboratory of the Microbiology Department of Karbala University, Iraq.

Serological evaluation

ELISA

The collected samples were centrifuged at 2500 rpm for 20 minutes then collected sera were stored at-20°C for 24 hours to detect IgG and IgM using Qualitative Sandwich ELISA. All processes for detection of anti-toxoplasma-specific IgM and IgG antibodies (Abs) were performed following the instructions of the commercial ELISA Kit (Sunlong®, China) (9). According to the instructions of the producer company, if the OD value is ≥ the cut-off, the result can be considered positive for toxoplasmosis. The collected sera were run in duplicates to confirm the accuracy of the findings.

Molecular evaluation

Preparation of parasites for DNA extraction

The placenta from 94 aborted women was cut into small pieces and then ground using a mortar and pestle (10). The DNA was extracted from the placenta according to the manufacturing company using Add Prep Genomic DNA extraction kit (Addbio®, South Korea). The extracted DNA from the parasite was measured using a nanodrop spectrophotometer. Nuclease-free water was used instead of DNA as control negative for PCR assay.

Nested PCR assay

Specific primer pairs were designed referring to the gene bank website (https://www.ncbi.nlm.nih.gov/) for the 529 repetitive *Toxoplasma* DNA sequences (MG574979.1). The nuclease-free water was used instead of DNA target as control negative in PCR Reaction to confirm the specificity of the primer design (Figure 1 and Table 1). PCR amplification was used to detect *T. gondii*. PCR amplifications were carried out in a 20µL reaction mixture containing 1x PCR buffer, 1.5 mM MgCl2, 200 µM dNTPs, 250 µM primers, and 1.5 U Taq polymerase. Two of the first outer primers (first primers) utilized in this reaction were the same primers used in the former gene amplification study (11) and recorded 412 bp, while the remaining two internal primers (second primers) were designed to obtain 323 bp (GenBank: MG574979.1, Figure 1 and Table 1).

CTGCAGGGAGGAAGACGAAAGTTGTTTTTTTATTTTTTTTTCTTTTTGTTTTTCTGATTTTTGTTTTTTTTGACTCG
GGCCCAGCTGCGTCTGTCGGGATGAGACCGCGGAGCCGAAGTGCGTTTTCTTTTTTTGACTTTTTTTTGTTTTTT
CACAGGCAAGCTCGCCTGTGCTTGGAGCCACAGAAGGGACAGAAGTCGAAGGGGACTACAGACGCGATGCCG
CTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGAAGGCGAGGGTGAGGATGAGGGGGTGGCG
TGGTTGGGAAGCGACGAGAGTCGGAGAGGGAGAAGATGTTTCCGGCTTGGCTGCTTTTCCTGGAGGGTGGAAA
AAGAGACACCGGAATGCGATCCAGACGAGACGACGCTTTCCTCGTGGTGATGGCGGAGAGAATTGAAGAGTG
GAGAAGAGGGCGAGGGAGACAGAGTCGGAGGCTTGGACGAAGGGAGGAGGAGGGGTAGGAGAGGAATCCAG
ATGCACTGTGTCTGCAG.

Figure 1. The DNA sequences for *Toxoplasma gondii* 529 repetitive sequences showing the outer primers are yellow color, while the inner primers are green color

Table 1. Nucleotide sequences for detection of toxoplasmosis by nPCR

Primer	Nucleotides sequences	Product/ References
First Forward	5-TTTTGACTCGGGCCCAGC -3	412 bp (11)
First Reverse	5-GTCCAAGCCTCCGACTCT-3	
Second Forward	5-GAGCCGAAGTGCGTTTTCTT-3	323 bp (Present study)
Second Reverse	5-ATTCTCTCCGCCATCACCAC-3	

qPCR for toxoplasmosis detection

To confirm nested PCR results, all positive samples by nPCR were confirmed by qPCR. In a final volume of 25 µl, The DNA template of forwarding primer was added to a reaction mixture containing 25 µl of 2 PCR universal master mix, and 5 µl B1 gene-F (5 M, 5′-TCCCCTCTGCTGGCGAAAAGT -3′), 5 µl of the reverse primer B1 gene-R (5 M, 5′-AGC GTT CGT GGT CAA CTA TCG ATT G-3′), with TaqMan probe (FAM-TCTGTGCAACTTTGGTGTATTCGCAG-TMRA) (10,11). The qPCRs were carried out using a GenAmp 5700 Sequence Detection System (Thermo Fisher Scientific). After activating AmpliTaq DNA polymerase for 10 minutes at 95°C as denaturation, 40 PCR cycles of 95°C for 15 seconds and 60°C for 1 minute were performed. The cycle threshold (CT), which represents the amount of target gene at which the fluorescence exceeds a predetermined threshold, was calculated.

Statistical analysis

Analysis was carried out using SPSS version 21 (SPSS, IBM Company, Chicago, USA). Categorical variables were reported as frequencies and percentages. Continuous variables were reported as means with their 95 percent confidence interval. Pearson's Chi-square test was utilized. The p value less than 0.05 was considered statistically significant using Duncan's Multiple Range Test.

Results and discussion

Of 94 aborted women hospitalized at Al-Diwaniyah Maternity and Pediatric Teaching Hospital in Iraq, 30 had a positive *Toxoplasma* PCR in their placenta (31.9%). Regarding the age of participants, 14 were younger than 20 years, 32 of them were between 20 and 30 years, and 48 were over the age of 30 years.

Based on the collected data, all patients were negative in the first trimester of pregnancy. Both ELISA and nPCR assays indicated that the positivity was respectively 8.51% and 6.06% in the second and third trimesters (Table 2).

The risk of parasites reaching the fetus depends mostly on the gestational age at the time of infection. Using both nPCR and ELISA assays, it was found that the prevalence of toxoplasmosis increased from 0% in the first trimester of gestational age to 8.51% in the second, and then decreased to 6.06 % in the third trimester, therefore, the results revealed that most of the abortion occurred at the second semester of gestational ages (Table 2).

Toxoplasma gondii and level of education

Considering the obtained results of the current study, it was found that an increase in the level of education in women was accompanied by a decrease in the rate of infection. The results were recorded as 29%, 19%, and 9% for women who finished primary, secondary, and high school, respectively (Figure 2).

Detection of Toxoplasma gondii in the experimental period using nPCR

According to the obtained result, there was no significate difference among different months of the study in terms of the rate of abortion. However, the cases of *Toxoplasma* reached their peak in May (62.5%) and April (60%) while there was no case in February (Table 3).

Table 2. Demographic characteristics of aborted mothers and the results of different diagnostic methods

Demographic data		nPCR		ELISA Anti-toxoplasma antibodies (Abs)				Both ELISA and nPCR	
		+	-	IgM^+	IgG^+	IgG^+ IgM^+	Abs -	+ (%)	- (%)
Age (Number)	< 20 Y (14)	5	9	1	3	1	9	1 7.1%	13 92.2%
	20-30 Y (32)	9	23	4	9	2	17	2 6.25%	30 93.75%
	> 30 Y (48)	16	32	5	13	3	27	3 6.25%	45 93.75%
Total	94	30	64	10	25	6	53	6	88
Statistical analysis		X2= 0.349 p > 0.05			X2= 0.671 p > 0.05			X2= 0.016 p > 0.05	
Gestational Age (Number)	First trimester (14)	2	12	0	5	0	9	0 0%	14 100%
	Second trimester (47)	17	30	7	11	4	25	4 8.51%	43 91.49%
	Third trimester (33)	11	22	3	9	2	19	2 6.06%	31 93.94%
Total	94	30	64	10	25	6	53	6	88
Statistical analysis		X2= 2.42 p > 0.05			X2=4.46 p > 0.05			X2=1.31 p > 0.05	

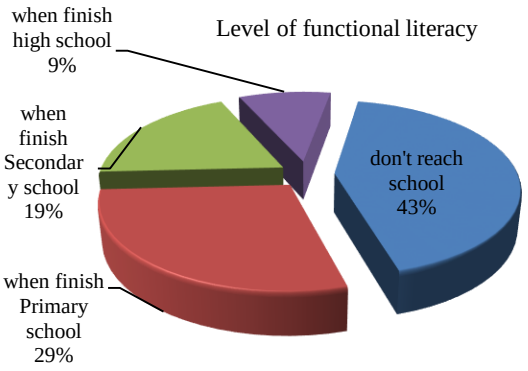

Figure 2. Prevalence of *Toxoplasma gondii* in women with different levels of education

Table 3. Prevalence of toxoplasmosis at the nine-point of time (month)

Date samples	No. of samples	No. of positive	Percentage
Dec-20	18	5	27.78
Jan-21	15	7	46.67
Feb-21	9	0	0
Mar-21	15	2	13.33
Apr-21	10	6	60
May-21	8	5	62.5
Jun-21	8	1	12.5
Jul-21	6	2	33.33
Aug-21	5	2	40
Total	94	30	31.9%
Statistical analysis		X^2= 8.98, p > 0.05	

Toxoplasma gondii and residential areas

As can be seen in Table 4, 53.3% of women in the rural areas were in contact with cats in Al-Qadisiyah

province. However, the case for women in the urban area was 23.3% (P <0.05).

Table 4. The number and percentage of toxoplasmosis in different residential areas

Residence	Characteristic contact of women with animal	Number	Percentage	Statistical analysis
Urban	Animal at home or in the neighborhood (N=21)	7	23.3	X^2= 3.591 P< 0.05
	No animal contact (N= 29)	3	10	
Rural	Animal at home or in the neighborhood (N= 35)	16	53.3	
	No animal contact (N=9)	4	13.3	
Total	94	30	100	-

Seroprevalence of toxoplasmosis via detection of anti-*Toxoplasma gondii* antibody using ELISA assay

The serology profile of the 30 women can be seen in Figure 3. As indicated, IgG was positive for 11 women (36.6%), IgM was positive for (10.63%), and 6 women (6.38%) were positive for both IgG and IgM. Among all, 3 women had a negative serology. All women having IgM or both IgG and IgM were positive by PCR assay. Three women carrying the parasite in their placentas (positive PCR) had a negative serology.

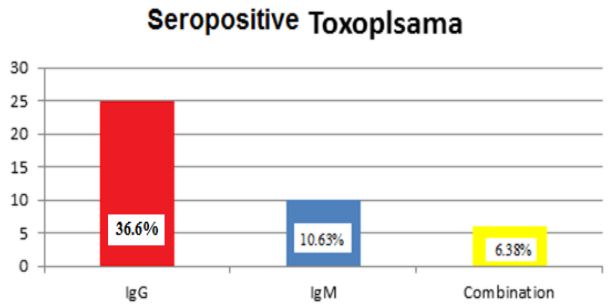

Figure 3. Prevalence of toxoplasmosis using ELISA assay

The presence of a unique band of DNA *T. gondii* at 412 bp was used to identify positive results for two first primers detection (Figure 4). The safest technique for ensuring PCR detection efficiency is to design second primers in conserved regions (529 repetitive regions) at 323 bp (Figure 5).

According to Table 5, the results of 11 women were positive for IgG and PCR method, and 10 women were positive for IgM and PCR technique. Six samples of women were positive for both tests. Among 53 negative samples, 3 samples were positive for the PCR test.

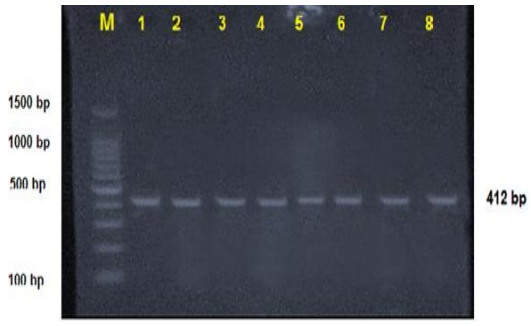

Figure 4. Nested PCR analysis of the 529 repetitive partial gene. M: Marker (100-1500bp), Lanes (1-8) positive samples at 412bp by PCR product

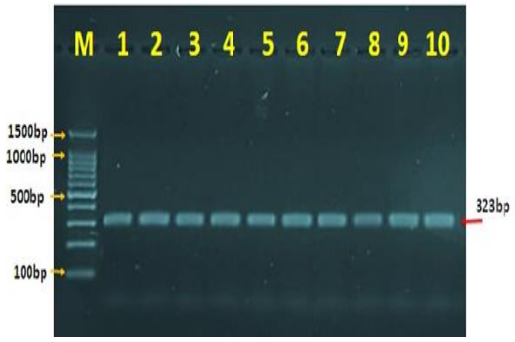

Figure 5. Nested PCR product analysis of the 529 repetitive partial gene. M: Marker (100-1500bp), Lanes (1-10) positive samples at 323bp by PCR product

Table 5. Comparing the results of ELISA and nPCR on detection of *Toxoplasma* infection

Methods		nPCR		No.
		+	-	
ELISA	Only IgG (+)	11	14	25
	Only IgM (+)	10	0	10
	IgG (+) & IgM (+)	6	0	6
	Negative	3	50	53
Total	-	30	64	94
Statistical analysis		X2= 52.62, P<0.05		

qPCR assay to confirm results of nPCR

The 30 samples positive by nPCR were also positive by qPCR (Figure 6).

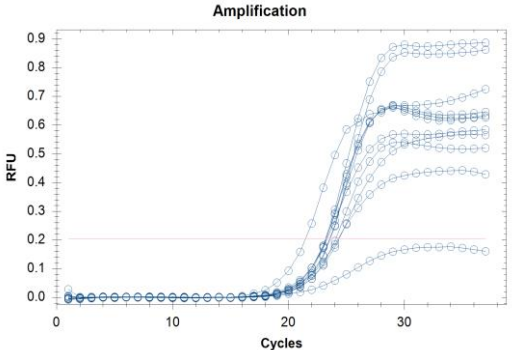

Figure 6. Amplification of qPCR samples. Positive samples crossed a threshold line. The samples below the threshold line are negative.

Abortion is a severe problem that any pregnant woman may confront (1). Among the 94 aborted women in the present study, 30 cases had a toxoplasmosis PCR positive test. Regarding epidemiologic characteristics, it was found that the infection gradually increased as women grew older. It is reported that the rate of fetal loss in women aged 20-24 years (9%) was lower than those aged 35 and over (75%, 12).

According to the obtained results, 53.3% of infected women who lived in rural areas were in contact with cats, confirming that the main origin of infection could be having contact with cats either directly or through eating vegetables contaminated with the oocyst. Contacting livestock in rural areas and eating different types of meat could also be other risk factors as people are used to eating roast meat (locally called Kebab) which is usually undercooked (12,13).

In the current study, the majority of cases of toxoplasmosis were detected in April (60%). A study on 40081 pregnant women indicated that the occurrence of acute toxoplasmosis during winter-spring was significantly more than summer-autumn (14). Although there are discrepancies in the findings on the relationship between time and toxoplasmosis, conducting more seasonal studies on pregnant women can determine the importance of different seasons on toxoplasmosis brilliantly (15).

Maternal infection increases the possibility of *Toxoplasma* parasites passing through the placenta to the fetus, which can cause congenital toxoplasmosis, and consequently abortion or fetal mortality (16). The severity of infection depends on the date of contamination during pregnancy (17). It is crucial to determine the immunological status of the pregnant woman, especially in high-risk areas.

Of the 94 women studied, 31 had anti-*Toxoplasma* specific Abs and 25 had only IgG Abs while 6 had both IgG and IgM. The presence of specific IgG and with/or in the absence of IgM Abs could point to a previous infection. However, the IgM Abs may be temporary or even negative in a recent infection (18). It is recommended to repeat the serology tests three weeks later to monitor any potential rise in IgG levels, which might indicate reinfection or reactivation (19). For the 10 women who had only IgM, there could be a possibility of a recent infection or a false-positive reaction. As a result, a two-week follow-up sample is required for confirmation of tests. Although there was no follow-up, these 10 women had a positive nPCR suggesting a recent toxoplasmosis infection.

PCR-based approaches for the diagnosis of toxoplasmosis have been established utilizing a variety of clinical specimens, including amniotic fluid (20), blood (21), and tissue biopsy (22). Among different diagnostic approaches, nested PCR has proven to be more sensitive. The real drawback of the technique is that it takes a long time and does not produce quantitative data. The real-time quantitative PCR technique has proven to be useful and can quantify the infection load of a clinical specimen. The entire 30 samples positive by nPCR using the G529 repeat gene were positive by qPCR using B1 gene primers. This method was based on a study in an ophthalmology clinic that utilized both nPCR and qPCR for the detection of *Toxoplasma* in patients with ocular injury and reported similar positive samples in both methods (23).

The percentage of *T. gondii* infection diagnosed by PCR in the present study was 31.9% of aborted women. The findings of the present study are different from those of previous researches (24,25), who respectively reported a relatively lower (6.4%) and much higher (80%) percentage of PCR positive among aborted women.

In terms of gestational age, the current study revealed no significant difference among the studied women. This was similar to the finding of a previous study on 201 pregnant women in Brazil indicating that the prevalence of the disease was not related to the age (26).

The number of cases detected with *Toxoplasma* was higher in the second and third trimesters of pregnancy in this study which was in accordance with a previous report (27). However, it is well known that abortion due to toxoplasmosis is more frequent in the first trimesters (28). This can be explained by the low number of collected placentae in the first trimester.

It was also found that three women carried the parasite in their placentas, and at the same time, they did not carry antibodies in their blood. The parasite passes through the placenta during the period of parasitemia following primary infection. The Abs appear after this phase, the parasites morph into the dormant cyst form, stay particularly in the brain and striated muscle tissue where they maintain the

protective immune status of the host. The negative serology in those three women could also be explained by the deficiency of the immune system in specific antibody production emphasizing the importance of the PCR to detect the etiology of abortion.

Conclusions

It is suggested that women in high-risk areas should be aware of the risk of toxoplasmosis and the importance of preventing measures. The prevalence of toxoplasmosis in the present study indicated that pregnant women should be tested during the pregnancy in areas with a high risk of infection. In addition, it is recommended to perform PCR in the case of abortion to enhance sensitivity even if the serological test result is negative. The findings of the present study were limited by the study area (a hospital-based cross-sectional study) and small sample size which probably missed some determinative epidemiological agents. Further studies on the detection of all effective infections and co-infections can show the real place of *toxoplasma* infection in spontaneous abortions

Acknowledgments

Not applicable.

Interest conflict

The authors declare that they have no conflict of interest.

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