

**Using Antibody rapid test, RT-qPCR, and RFLP/PCR for detecting
Toxoplasma gondii in the placenta of abortion women and
determining the genotype in Maysan province, Iraq .**

Safaa Salman Allamy, and Hussain A. Mhouse Alsaady

University of Misan, College of Science, Department of Biology, Maysan, Iraq.

Abstract

Toxoplasma gondii is one of the obligatory intracellular protozoan parasites that are important and widespread in all developing or developed countries. It infects all nucleated cells of warm-blooded animals. This study used the lateral flow immunoassay (LFIA), real-time quantitative polymerases chain reactions (RT-qPCR) for detecting the *T. gondii* in blood and placental tissues, in addition to the restriction fragment length polymorphism (RFLP) technique to determine the genotypes/strains of *T. gondii* in Maysan province. The study included 40 participating women, distributed as 22 aborted women and 18 without abortion. The LFIA detected that the seropositive rate of anti-*T. gondii* IgG is 38.9 % and with the using RT-qPCR technique, the infection rate is 10 % and 0.0 % among placenta and sera. it also, showed that 85.7 % of all *T. gondii* infections are found in aborted women, compared with 14.3 % without abortion. In contrast, it showed that 63.6 % of *T. gondii* seronegative are women without abortion, compared to 36.4 % among aborted women. The PCR-RFLP technique has identified the main three genotypes “I, II, and III” of *T. gondii* in Ammara city, and showed that genotype "I" is the most frequent at 50% (6/12) than genotypes II, 4/12 (33.33 %), and III, 2/12 (16.67 %).

Keywords: *Toxoplasma gondii*, LFIA, RT-qPCR, RFLP, genotype, abortion women, Maysan, Iraq

1. Introduction

Toxoplasma gondii is an intracellular coccidian zoonotic obligate parasite [1]. This

parasite was described for the first time in the tissues of the desert rat “*gondi* (*Ctenodoactylus gundi*)” at the Pasteur

Institute in Tunis by Nicolle and Manceaux synchronously with Splendore in rabbit in Brazil. The full life cycle remained unknown until 1970 when the cat was discovered as the definitive host [2-4]. It infected the nucleated cells of about 350 endothermic animals as intermediate host [5]. Globally, *T. gondii* occupies the fourth rank in terms of importance among foodborne parasites [6]. *T. gondii* infects humans and other animals through one of the main stages of transmission mature oocysts, tachyzoites, and bradyzoites [7]. Initially, the tachyzoite invades the host cells but prefers the monocyte set, especially macrophages, it avoids the phagocytosis processes by creating parasitophorous vacuoles [8, 9] as a shelter for growth and multiplication. Until the cell exploded, and the parasite attacks the rest neighboring cells, forming lytic cycles, which increases pathogenesis, by exploiting the macrophages as a Trojan horse to increase its spread by transmission to other tissues [5, 10].

During the acute phase of the infection, which does not exceed ten days, the parasite faces a strong immune response against its spread throughout the body, but it avoids this immune response by turning to the inactive stage (Bradyzoites) in cyst inside the muscles or brain [11]. The parasite can

avoid the body's immunity by turning into an inactive phase and within the muscles and brain tissues. The parasite is responsible for causing birth defects such as congenital toxoplasmosis and abortion of pregnant women [12]. The early treatment of toxoplasmosis can reduce depression in pregnant women, abortion cases, and the economic impact of the parasite, such as the treatment cost of infected infants who suffers from blind and mentally retarded [13-15]. It is necessary to raise awareness and early diagnosis of toxoplasmosis with highly sensitive tests, like Polymerase chain reaction (PCR) or serological tests [16]. The worldwide distribution and knotted life cycle, including the sexual stage, enabled the parasite to recombination its genes, which led to the emergence of a wide variety of genotypes in the world [17].

Molecular studies estimate that there are more than 200 genotypes of *T. gondii* spread in different regions of the world, but there are three genotypes of which, I, II, and III are predominant [18]. The importance of studying genotypes is returned to their relationship with epidemiological and clinical complications of toxoplasmosis [19], epidemiological and clinical studies. The dominant genotypes are distributed worldwide, in Europe is found that the

dominance is genotype II, which is often associated with congenital toxoplasmosis [17, 20]. In Africa, the dominant genotypes are II, and III, while in Asia, genotype I [21]. Genotype I is responsible for ocular toxoplasmosis [22]. The three dominant genotypes I, II, and III are distributed worldwide and isolated from humans, they varied in their virulence and epidemiological patterns [23]. In humans, genotype I is mainly shown in cases with ocular toxoplasmosis and causes serious toxoplasmosis [24]. Genotype II is related to chronic incessant tissue cysts [25]. Type III is an unusual human genotype, but it is the most isolated strain from animals [26]. The three dominant genotypes I, II, and III are distributed in various` regions of the world, they were isolated from humans and varied in their virulence and epidemiological patterns [23]. In humans, genotype I is mainly related to ocular toxoplasmosis and causes serious toxoplasmosis [24]. Genotype II is related to chronic incessant tissue cysts [25]. While genotype III is uncommon in humans and often isolated from animals [26]. In Europe, the dominant genotype is II [17, 24], and genotype III comes in the second rank. In Africa, the dominant genotypes are II and III, and in Asia, genotype I [21]. The PCR-RFLP is the most successful and accurate technique

in distinguishing between *T. gondii* genotypes [27]. The Antibody rapid test, LFIA is easy and simple in design, small and compact, with the possibility of interpreting the results and rapid test within minutes, with the possibility of testing whole blood [28, 29].

2. Materials and Methods

2.1 Tissue samples

The samples of blood and placentas tissues were collected from the participant women in this study from Al-Sadr Teaching Hospital during the period from Nov. 20, 2021, to May 19, 2022. Five ml of venous blood from each woman was collected and placed in an EDTA tube and gel and clot activator tubes. the placenta samples were cut into 0.5×0.5 cm pieces, washed carefully, and then preserved in 10 % formaldehyde. According to the company's instructions, the DNA of *T. gondii* was extracted from the placenta and sera samples by using the AddPrep Genomic DNA Extraction Kit (Addbio, South Korea).

2.2. Lateral Flow Immunoassay (LFIA)

The LFIA kits, OnSite®Toxo IgG/IgM Combo Rapid Test (CTKbiotech,

USA) are used to detect the anti-T.gondii IgG and IgM in sera of the participants women. Briefly, the blood samples were left in the gel and clot activator tubes for 15 min at room temperature. The sera were separated by centrifugation the blood samples at 3000 rpm for 10 min. LFIA strips were labeled with the code number of the blood samples and placed horizontally on a clean, flat surface, then using the micropipette add 10 µl of serum into the well of the strip cassette, and immediately add 2 drops of the sample's diluent on top of the serum. The strip was left for 10-15 min on a flat surface without any airflow. In this period, the serum migrates laterally by capillary action to the conjugate pad containing T. gondii antigen and a control antibody conjugated with colloidal gold. If the serum contains anti-T. gondii IgM, or IgG, it will bind to Toxoplasma antigen to form an immune complex that is picked up by anti-human IgG pre-coated on the line. If the red color appears at the IgM line, this means the serum is positive and the Toxoplasma infection is acute, and if at IgG line indicates chronic infection, but if only a red line appears on the control line, this means the sample is negative for toxoplasmosis. The red color and intensity the color depends on the number of immunoglobulins present in the blood sample.

2.3 RT-qPCR

Some samples were used in the implementation of the probe-based RT-qPCR. A specific probe (FAM-5'-CCACCTCGCCTCTTGG-3'-BHQ1) and one pair of specific primers, (forward-3'-CTAGTATCGTGCGGCAATGTG-5' and reverse-5'-GGCAGCGTCTCTTCCTCTTTT-3') were used in the amplification process of the conserved region of Toxoplasma gondii gene B1 [30]. Use 2 ng of genomic DNA and GoTaq® Probe RT-qPCR Master Mix (Promega, USA) and the reactions were performed in 0.2 µl wells, with a total volume of 20 µl. The wells were placed in an RT-qPCR thermal cycler (Stratagene, USA) at 95 °C for 10 min followed by 40 cycles of "95 °C for 15 sec and 60 °C for 1 min". The amplification success rate was better than 95 % and 1 % as error rate calculated on PCR duplicates.

2.4 RFLP/PCR

The infected placenta was Chopped into small pieces of 0.5 × 0.5 cm, then washed, and then homogenized with phosphate-buffered saline at pH 7.2. The DNA of T. gondii was extracted from homogenized placenta by using the AddPrep Genomic DNA Extraction Kit (Addbio,

South Korea), and according to the company's instructions that attached with kit. The genotypes identification of the Ammara isolates of *T. gondii* were carrying out by using the PCR-RFLP analysis of the gene GRA6 as explained by Norouzi et al. [31]. Using two primers of the gene GRA6, (forward-3'-

GTAGCGTGCTTGTTGGCGAC-5' and reverse-5'-

TACAAGACATAGAGTGCCCC-3') which amplifies an 773 bp DNA fragment of GRA6 gene extracted. The PCR was done by taking 4 µl of each DNA samples and 40 umol of each primer to obtain a final volume of 40 µl. Then, each extracted DNA of infected placenta was amplified under the thermal conditions: initial denaturation for one cycle, at 94 °C for 5 min, followed by 35 cycles, each, denaturation at 94 °C for 30 sec, annealing at 56 °C for 30 sec and extension 72 °C for 1 min, and a final extension for one cycle at 72 °C for 5 min. Restriction digests were prepared in a final volume of 40 µl from 3 µg of each PCR products with a 1 µl (10 units) of enzyme restriction endonuclease MseI and restriction buffer 5 µl (1X) of NEBuffer obtained from NEB (New England Biolabs, USA), with total reaction volume of 40 µl by DDW for 4 hours. After that, the digests were incubated for 3 hours at 37 °C.

The products of restriction fragments of *T. gondii* were electrophoresed through a 1.0 % agarose gel at 90 volts for 45 min (Electrophoresis cell, Cleaver UK) with using 5 µl of Safe-Green 100 bp Opti-DNA Marker(ABM, Canada) in first well.

3. Results and Discussion

3.1 Lateral Flow Immunoassay

The results of the immunoassay, LFIA exhibited that 38.9 % (7/18) of women are seropositive for anti-*T. gondii* antibody. This finding is in line with some findings in other countries such as Saudi Arabia, 38 % [41], Sri-Lanka, 37.8 % [32]. It is less than that found in Nineveh, northern Iraq, 73.33 % [33], in Maysan southern Iraq 52.3 % [64], and it is higher than that found in some regions of the world such as Saudi Arabia 21.3 % [34], and Bangladesh 25 % [35]. Moreover, other recorded cases in some province of Iraq like Baghdad 18 % [36], Anbar, 13 % [37], Salahaldeen 30 % [38], Babylon 34.28 % [39], and in Maysan 20 %. Table 1 revealed that 85.7 % of all seropositive of anti-*T. gondii* IgG antibody are among aborted women, compared with 14.3 % of without abortion. At the same time, it shows that 60 % (6/10) of all abortions were found to be seropositive of anti-*T. gondii* IgG, compared to only 12.5 % (1/7) of

non-aborted women. In contrast, it showed that 63.6 % of *T. gondii* seronegative are among women without abortion, while there is about 36.4 % of *T. gondii* seronegative are among aborted women. The statistical analysis of the current data indicated a significant relationship between toxoplasmosis and the abortion of women ($\chi^2= 4.219$, $P<0.05$). In other hand, it shows that all seropositive women had only anti-*T. gondii* IgG, and non-one had anti-*T. gondii* IgM. Same results were recorded in Aydin, Turkey [40], in Riyadh, [41], in Zambia [42], and in United States [43]. The absence of anti-*T. gondii* IgM indicated that all infections are chronic [44]. Because the level of IgM rises during the first two weeks of infection and then gradually decreases and replaced by IgG. The present anti-*T. gondii* IgM or IgG or both can help distinguish between acute and chronic infections [45].

Table 1: The rate of anti-*T. gondii* IgG among the abortion and non-abortion women by LFIA.

Anti-Toxoplasmosis		abortion			χ^2	P
		Having	Without	Total		
		No. (%)	No. (%)	No. (%)		
IgG seropositive	No. (%)	6 (85.7) (60.0)	1 (14.3) (12.5)	7 (100) (38.9)	4.219	0.04
IgG seronegative	No. (%)	4 (36.4) (40.0)	7 (63.6) (87.5)	11 (100) 61.1		
Total	No. (%)	10 (55.6) (100)	8 (44.4) (100)	18 (100) 100		

3.2 Real time PCR for blood

Forty blood samples including 22 aborted women and 18 without abortion are analyzed by RT-qPCR technique to detect the DNA of *T. gondii* in these two groups. The RT-qPCR technique could not identify the DNA of *T. gondii* in any blood sample of aborted or non-aborted women. The finding of this study is corresponding to the findings of Chiang et al. [46] in Taiwan who showed no Toxoplasma-DNA was detected by RT-qPCR technique. Closely like the findings in Turkey who found that the rate was (1.3 %) and with Aziz [60] in Wasit province who found that RT-PCR detected only 2 % of 300 blood sample of sheep compared to Latex test which found that 100/300 cases are infected with *T. gondii*. Al-Mosawi et al. [47] found that RT-qPCR detected only 6.26 % while the

ELISA detected 21.94 % within same group. Nakashima and colleagues found only 10.8 % of the 353 ELISA-seropositive cases were detected in PCR and the rest of the positive cases (89.2 %) were PCR-negative [48]. Some attributed this disparity in this assay as resulting of the effect of the non-specific DNA which inhibits the PCR in the Light Cycler tool Nakashima et al. [48], and the decline in PCR sensitivity may be due to the amount of template existent at the initiation of the PCR reaction, this affects the cycle threshold value, if the amount of template is high, the reaction needs to few-cycles to form enough product and low reaction while in lower amount need more amplification cycles and need a long period of reaction Carr and Moore [47, 49]. For this the accumulation of PCR products at a certain point led to a huge loss in their efficiency [49]. But this finding disagrees with Al-nasrawi et al [50], and Shaker et al. [51] who found that 16 % and 15 % respectively of aborted women had positive Toxoplasma-DNA by RT-PCR. The decline of the sensitivity of RT-qPCR in detection Toxoplasma may be attributed to the parasite's behaviour. It showed at the acute period the active stage "tachyzoite" is quickly disappearing inside monocytes in a special structure "Parasitophorus vacuole" [8], and during the first ten days of infection,

the body exhibits an acquired immune response against the tachyzoite.

Under these circumstances, the tachyzoite is not able to confront the body's immunity, so it is forced to escape from blood to hide in the muscle or nervous tissues which take them as a haven from attack the immune elements and turns to inactive-hypozoite "bradyzoite stage" in tissue cyst for this reason the declining number of this parasite in the blood can be noticed. The absence of *T. gondii* DNA may be attributed to the rare presence of this parasite in blood, and if it is found, this is for a short time [52]. As well as the ability of the immune system to clear any foreign materials in the blood, such as debris, large molecules, or remains of parasites such as DNA [53]. In these circumstances, it is difficult to detect this parasite by PCR [52].

3.3 Real time PCR for placenta

The primers amplified 62 bp DNA fragments of the B1 gene in the technique of RT qPCR. The analysis of *T. gondii* DNA of twenty placental tissues of aborted women revealed only (10 %) of women's placentas are infected with toxoplasmosis in table 2, and figure1.

Table 2: RT-qPCR test for placental tissue samples.

Toxoplasma DNA		
Number	Positive (%)	Negative (%)
20	2 (10 %)	18 (90 %)

The cycle threshold value of RT-qPCR for the two positive cases ranged between the cycle 25 and 26 (figure 1). There is more than one study facing the same problem such as Filisetti et al. [54], who found of 785 placenta was 2.03 % are positive in PCR. The low of RT-qPCR sensitivity may be attributed to the amount of template which is found at the initiation of the PCR reaction [49]. This finding is less than what found by Al-Dujaily and Abdul-Amir [55] 50 % of aborted women, Abbas et al. [56] (17.7 %). The low sensitivity of this technique may be due to this technique did not capable to detect or sense the parasite in the macrophage and in tissue cyst [57].

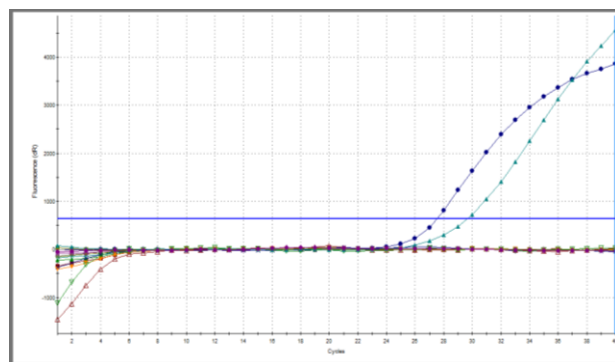


Figure 1: RT-qPCR analysis of the placental tissue.

3.4 Detection of *T. gondii* genotypes

The *T. gondii* genotypes are identified by RFLP-PCR. The present study discovers the presence of the three main genotypes of *Toxoplasma* parasite, I, II, and III in the placentas of aborted women in Maysan province southern Iraq, and the frequency of the I genotype was the most common among all the other genotypes, with a frequency of 50% (6/12), then genotype II with a 33.33%.

Table 3: The genotypes of *T. gondii* among placenta of aborted women by PCR-RFLP assay.

Genotype	No. of isolations	Frequency %
I	6	50.00
II	4	33.33
III	2	16.67
Total	12	100

This finding is consistent with what Al-Hadraawy et al. [58], who found in their study in Najaf province where they found I, II, and III with the frequency of 60 %, 25 %, and 15% respectively. These results disagree with findings in other locations of Iraq such as Mohammed et al. [59] in Baghdad, and Aziz [60] in Wasit who found the dominant genotype is II, and Al-Khanak, and Salman [63] in Wasit who found that genotype III is the dominance.

In Egypt, Abdel-Hameed and Hassanein [61] found that genotype II is the most frequent at 87 % and genotype I at 13 % but they do not find genotype III. The variation in the distribution of the genotype may be due to the difference in climatic conditions and dietary habits and may be related to some extent to the import of meat or livestock from other countries that may be endemic with other genotypes [62].

The diversity of *T. gondii* genotypes in Iraq may be associated with more than one reason, including what is related to the role of Iraq historically, as it was the capital of many states that were established successively and a focus for the attention of scholars. Iraq has been subjected to a foreign regional and international invasion from the dawn of history, where the invaders enter with their various animals, bringing with them the diseases that are endemic in their countries. It was subjected to invasion and occupation by the Persians, Seljuks, Tatars, Ottomans, and then the British, along with the Indians. Iraq was the scene of the first and second world wars. Then the American invasion during the past three decades in the First, and Second Gulf wars, all these brought the parasites and transitional diseases. In addition to trade that is not subject to quarantine laws and the trade in foodstuffs and pets such as cats, dogs, rat,

birds, and monkeys from different regions of the world. This trading caused to transmission of European strains II, III, African, and Asian strains. In addition, Iraq possesses large water bodies represented by the marshes, whose area is estimated at 37,9177 Km² which encouraged the annual visits of migratory birds, which constitute one of the means of spreading *Toxoplasma* strains.

3.5 Ethics Approval and Informed Consent

Ethical approval for this study was obtained from the ethical review board of the Misan Health Directorate with reference No. 211 on 12/2/2021 .All participants were duly informed of the objectives of the study and the protocol for sample collection. All participants signed an informed consent form were signed. Participation was voluntary.

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