

Molecular detection of *Toxoplasma gondii* specific repeat element in blood of recurrent aborted women by real-time PCR

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ABSTRACT

Toxoplasmosis caused by ingestion of raw or undercooked meat contains tissue cyst or contaminated food or water with oocyst of *Toxoplasma gondii*. This parasite is prevalent in humans, domestic and wild animals and is causative agent of abortion and congenital abnormalities. Methods: From October-2019 to April 2020, blood and serum samples were collected from 63 women with recurrent abortion. Serum samples were used for identification of anti-*Toxoplasma* IgM and IgG antibodies by ELISA technique. Real time- Polymerase Chain reaction technique utilized for revealing of the DNA of *Toxoplasma gondii* in the blood specimens. Presented study results showed the high aborted women 33 (52%) in age group (20-29), and high number of aborted women 36 (57%) have 3-6 abortions. Serodiagnosis results of 63 aborted women showed that 19 (30%) were seropositive for only IgG antibody, 6(10%) were seropositive for IgM antibody, and 6 (10%) were positive for both IgG and IgM antibodies. Molecular diagnosis of *T. gondii* DNA infection by RT- PCR for amplification of repeat region 529 bp of *T. gondii* DNA, revealed that only 7 (11.1%) blood samples were positive. RT-PCR assay was more sensitive and specific than serologic ELISA method in diagnosis of *Toxoplasma gondii* infection in recurrent aborted women.

Keywords: *Toxoplasma gondii*, ELISA, RT- PCR, repeat region element.

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1. Introduction

Toxoplasma gondii, commonly found to be one the zoonotic infectious parasite, was first reported in a North African rat by Nicolle and Manceaux in 1908, and causes toxoplasmosis in humans, domestic and wild animals [1, 2]. Approximately one-third of the population was affected from this protozoan [3]. Human infections occur through the consumption of meat containing viable tissue cysts from undercooked or raw meat or through the ingestion of water or food contaminated by *Toxoplasma. gondii* oocysts. In approximately 25-30% of cases, *T. gondii* may be transmitted via the placenta to the fetus and causes abortion and congenital abnormalities, like stillbirths , longstanding debilitating sequelae, or death of fetus [4]. The severity and frequency of congenital toxoplasmosis counts on the gestational period of the acquired maternal infection. The onset of the infection is often asymptomatic, and most of the infected individuals show no symptoms. In certain populations, the infection can result in severe illness and death, including encephalitis, chorioretinitis, congenital infection, and early pregnancy neonatal mortality [5]. In epidemiological studies, different serological and molecular methods have been employed by researchers worldwide to detect *Toxoplasma gondii*. Previous studies primarily focused on serological tests to identifying the *Toxoplasma*-specific IgM and IgG concentrations in the serum of patients. Serodiagnosis and quantification of *T. gondii* infection which can last for months or even years after acute infection in pregnant women, so these methods cannot distinguish recent infections from previous infections [6, 7]. Different molecular methods used in clinical sampling to assess the existence of parasites. Molecular diagnosis is sensitive, specific and used for early diagnosis of congenital toxoplasmosis and active pregnancy toxoplasmosis. The examination of *Toxoplasma. gondii* by RT-PCR has been utilized to detect parasites in tissues of animal and in amniotic fluids or whole blood from

human clinical specimens [8, 9].

The various target genes used in the detection of *T. gondii* was first created by Burget Al. (1989) using the molecular process B1 gene. High sensitivity has been shown by quantitative PCR, which targets repetitive DNA sequences [10] The single copy gene P30, the replicated B1 and the 529-bp repeat factor (AF146527) are also used. Earlier studies have revealed that multi-copy target genes are more liable to detect *T. gondii* as opposed to those that showed single-copy targets [11, 12]. There are 2 typical gene targets utilized the AF146527 sequence and 35-repeat B1 gene, a portion which is copied in the genome about 200- 300 times. Even though the accuracy of the tests with above genes has been already shown, the specificity stays the focus of additional study through utilizing a larger number of *Toxoplasma. gondii* strains [11]. Thus molecular methods are important for the initial identification of congenital and active pregnancy toxoplasmosis [13]. The presented study aimed at detection of anti-Toxoplasma IgG and IgM antibodies in serum by using ELISA, and finding of *T. gondii* DNA in peripheral blood in women who had frequent abortion referred to the Azadi teaching hospital in Kirkuk city by RT-PCR and using repeat region element as a target gene.

2. Materials and methods

This study conducted on 63 women have recurrent abortions attended to Azadi Teaching Hospital from October-2019 to April 2020, their age ranged 19-42 years. Questioner designed for patient's data collection, and included the following information: age, number of abortion, educational status, occupation, residency, gestation time, abortion trimester, clinical symptoms, contact with cats, blood transfusion, contact with soil in gardening and agricultural activities, and consumption of raw and undercooked meat, unpasteurized milk or raw vegetables.

2.1. Blood samples

Five ml of venous blood samples were collected in a sterile serum tube, three ml of the collected blood were left 30 minutes at room temperature, then the serum was divided and kept at -20 °C until the examination time. Anti-Toxoplasma IgM and IgG antibodies identified by using a commercial ELISA Kit. The assay carried out according to the manufacturer's instructions. The remaining 2 ml blood sample were placed in anti-coagulant tube with sodium citrate, the buffy coat collected after centrifugation of whole blood for 10 minutes at 1500 round per mint. and isolation of plasma. Buffy coat then stored in 70% ethanol at -20 °C for DNA extraction.

2.2. Determination of anti-Toxoplasma IgM and IgG antibodies in serum by ELISA

Using the commercially available ELISA kit, anti-toxoplasma IgM and IgG antibodies were identified in patient sera (Bioactiva Diagnostica GmbH, Germany, catalog no. TOXG01, and catalog no. TOXM02). The reaction was carried out based on the orders of the manufacturer. Using an ELISA plate reader at a wavelength of 450 nm, the optical density (OD) was measured. The results were qualitatively tested by measuring the ratio between the sample's average optical density value and the cut-off value. The specimen stated positive for the occurrence of specific antibodies when the absorption of the sample was greater than that of the Cut-off (the ratio is >1.1 for IgG and >1.1 for IgM). If the ratio is < 0.9 for IgM, and < 0.9 for IgG, the specimen is measured as negative.

2.3. Molecular diagnosis of *Toxoplasma gondii* by real time-polymerase chain reaction technique

DNA extraction

Toxoplasma. gondii genomic DNA extracted from blood sample using a RealLine Pathogen Diagnostic Kits (BIORON Diagnostics GmbH, Germany). The extracted DNA kept frozen at -20 °C until use.

2.4. Amplification of *T. gondii* repeat region element

The Real-time PCR was performed for detection of *T. gondii* DNA from blood samples of 63 selected women who have had recurrent abortions by using Techne qPCR test kit (Techne™ is a trademark of Bibby Scientific Ltd.) targeting Repeat region element. The primers used in this kit have designed based on *T. gondii* repeat region 529 bp that to have reference genomic sequences in the NCBI database (AF146527, EF195646.1, and AF487550.1.). The primer and probe mixture supplied takes use of known as TaqMan® principle. Positive control, negative control, and internal control were included in the each run PCR. A negative control represented by distilled ultrapure water.

2.5. RT- PCR program

PCR was carried out in a 15 µl reaction mixture consisting of 12 µl of 2x qPCR Master Mix, 1 µl of *T. gondii* primer/probe, 1 µl of internal extraction control primer/probe, 3 µl of RNase/DNase free water. Optimum PCR cycling parameters were; enzyme activation 95 °C for 2 mints, 50 cycles of denaturation at 95 °C for 10s and 60 °C for 60s were performed.

2.6. Statistical analysis

The Chi squared test was used to determine the association between; the age and infection, number of abortion and infection, seropositive cases of IgG and IgM, and number of positive cases detected by ELISA in comparison to number of positive cases detected by real- time PCR. The P value < 0.05 was considered statistically significant.

3. Results

3.1. ELISA results for *T. gondii* IgM and IgG antibodies

Current study conducted on 63 women who have recurrent abortions, their age ranged between 19-42 years. According to distribution of recurrent aborted women among age groups, results showed that the high aborted women 33 (52%) was in age group (20-29), where less aborted women 3 (5%) were found in age group (40-49) as shown in Table (1).

Table 1. Distribution of recurrent aborted women among age groups

Age- groups (years)	No.	(%)
20-29	33	52
30-39	27	43
40-49	3	5
Total	63	100

In regarding to the number of abortions among aborted women, the findings of the study revealed that high number of aborted women 36 (57%) have 3-6 abortions where 7(11%) women have 7-8 abortions, as shown in table 2.

Table 2. Number of abortions among aborted women

Variables	Aborted women	
	No.	(%)
Number of abortion	2	32
	3-6	57
	7-8	11
Total	63	100

As shown in table (3), 63 aborted women were diagnosed with anti-toxoplasma antibodies in serum by using ELISA technique. Of these, 19 (30%) were seropositive for only IgG, 6(10%) were seropositive for IgM, and 6 (10%) were positive for both IgG and IgM.

Table 3. Anti-Toxoplasma IgM and IgG antibodies in aborted women

Anti-Toxoplasma antibody	Aborted women			
	Positive		Negative	
	No.	(%)	No.	(%)
anti-Toxoplasma IgM	6	10	57	90
anti-Toxoplasma IgG	19	30	44	70
Both anti-Toxoplasma IgM and IgG	6	10	57	90

Chi-square = 13.042, P-value = 0.0014722

3.2. RT-PCR amplification of repeat region element

Sixty-three blood samples were subjected to real-time PCR for detection of *T. gondii* DNA infection. Molecular amplification of repeat region (EF195646.1, AF487550.1, AF146527) of *T. gondii* DNA, revealed that only 7 (11.1%) blood samples were positive by RT-PCR (Figure 1).

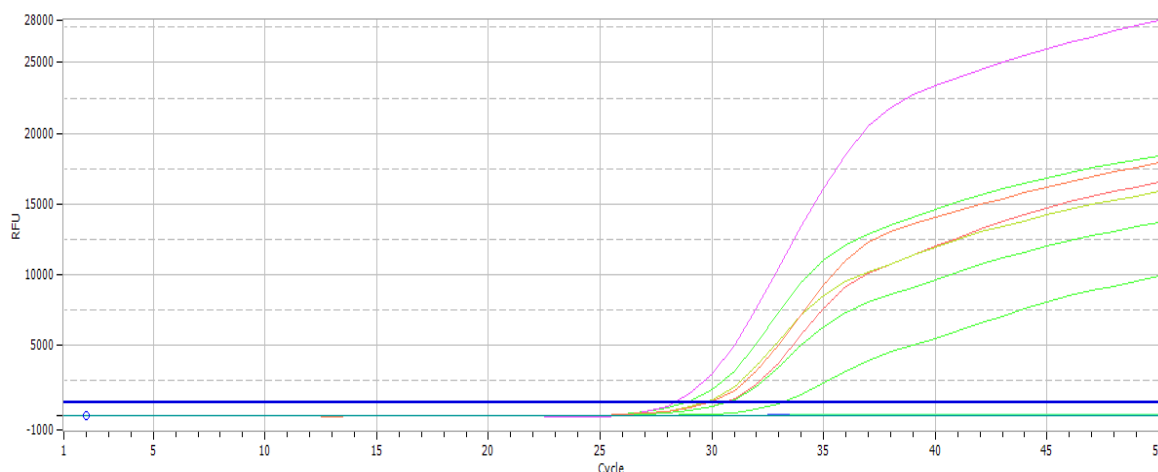


Figure 1. RT-PCR analysis of *Toxoplasma gondii* genomic DNA using repeat region (EF195646.1, AF487550.1, AF146527) in aborted women.

Comparison between RT-PCR and ELISA assays, results showed that 6 of 63 blood samples were found to be positive for both RT-PCR and IgM, IgG ELISA; three of RT-PCR positive cases was IgM positive and the remaining three cases were IgM seronegative ($P < 0.001$). Four of RT-PCR positive cases were IgG positive and the remaining were IgG seronegative ($P < 0.000005$), and one case was positive for both IgM and IgG. On the other side, RT-PCR detected one positive case that could not be detected by ELISA against IgM and IgG, as shown in table (4).

Table 4. Comparison between ELISA and RT-PCR results for toxoplasma in recurrent aborted women

Anti-toxoplasma Antibody		PCR		Total (n=63)	P-value
		Positive(n=7)	Negative(n=56)		
Anti-toxo IgM	Positive	3	3	6	0.001
	Negative	4	53	57	
Anti-toxo IgG	Positive	4	2	6	0.000005
	Negative	3	54	57	

3.3. Discussion

Toxoplasma gondii, is strictly intracellular parasite that invade vertebrates, specially humans and causes what so-called toxoplasmosis. *T. gondii* allocated especially among human population, also more than a billion people worldwide believed to be affected. Farm animals are imperative reservoirs of *Toxoplasma gondii*, this is critical because of the possibility of human transmission and leads toxoplasmosis. This parasite is highly susceptible to two major subpopulations: the fetus and an individual who is immunocompromised. Congenital infection may cause spontaneous abortion or serious complications during pregnancy when a maternal infection occurs. The damage to the fetus and the frequency of trans-placental transmission typically depend on the stage of pregnancy [6, 14, 15]. The detection of *Toxoplasma gondii* in infected patients is built on serological techniques to detect IgG, IgM, and IgA antibodies. There are many methods of serology that could be used. The ELISA technique is more sensitive, automated, precise, and convenient. However, in pregnant women, seropositivity for anti-*Toxoplasma* Abs is one of the most complicated situations [6]. In present study, the results showed significantly high IgG serum positive cases in aborted women, while less number of IgM, and both IgG and IgM serum positive cases found by using ELISA. The serologic tests are almost used

for detection of anti-Toxoplasma (IgA, IgM, IgG) antibodies instead of antigens by using enzyme-linked antibodies [16]. First antibody in serum occurs in women infected with *T. gondii* is IgM, by beginning of the infection [17]. IgM antibodies are the most effective complement system activators in humans. They promote good agglutination due to their high degree of cytotoxicity and its structure; this responsiveness is used predominantly in serological diagnostic methods. In toxoplasmosis, a second Abs tends to be IgG, several types of IgG are revealed by *T. gondii* infection comprising IgG1, G2 and G3. Because of the capability of IgG to cross the placenta, they have an important role in fetus protection. A shallow antigens of *T. gondii* are the main objective antigens for IgG. This rise in the amount of IgM is related to growth in the number of B-Cells [18, 19]. The presented study agreed with results of previous studies, such as results of study conducted in Saudi Arabia, revealed a greater incidence of IgG antibodies (38 %), and IgM was 0%, [20]. Another research in Qatar showed that 5.2% and 35.1% of pregnant women confirmed positive for IgM and IgG, respectively. Also similar to results of study done in Saudi Arabia among pregnant women that found the incidence of *T. gondii* IgM and IgG antibodies was 6.4% and 32.5% respectively. Other studies published by East Azerbaijan/ Iran, the IgG antibodies seropositivity ranged from 35.1% to 38.66% [21]. In ELISA assay, IgM found 9 (6.5 %) of the cases to be positive for immunoglobulin M, with 128 (93.5%) negative. Immunoglobulin G detection findings were positive in 53 patients (38.6 %) and negative in 84 cases (61.4 %), [22]. In Libya, from 140 women with recurrent abortion; 36(66.6 %) were seropositive for IgG [23]. Other findings observed significantly high IgM serum levels in women with abortion history and had *T. gondii* infection, explaining that the elevation in serum levels of IgM resulting the acute stage of Toxoplasmosis and increasing the avidity of IgG leading to the chronic stage of the infection [24]. There are many reasons that may clarify this difference in the seroprevalence rate. It is commonly proposed that the presence of particular IgM is correlated with acute infection. In addition, the regional differences in prevalence have been related the environmental, and the traditional distinctions in the type and amount of consumed uncooked meat and the raised in meat intake from livestock's farm and freezing meat. The occurrence of *Toxoplasma gondii* in humans differs from an area to another area, with ratios ranging lower than 10% to greater than 90% [25]. In study done in France positive cases for IgG-*T. gondii* antibodies by using ELISA was 47%. The seroprevalence of *Toxoplasma gondii* differs greatly across regions in Turkey, ranging from 30.7% to 69.5% [26]. Just 16 percent of individuals aged from 12- 49 years old showed positivity for toxoplasmosis serology test, in the United States. However, due to improved hygiene standards and frequent freezing, the seroprevalence of *Toxoplasma gondii* in developed countries is usually lesser which involves in the destruction of cysts in contaminated meat. The present study results of *Toxoplasma gondii* DNA amplification by RT-PCR using repeat region element showed that 7(11.1%) blood samples of total 63 samples were positive. Previous studies showed that the *T. gondii* DNA could be identified by PCR in women's blood samples before and during the pregnancy [27]. The existence of *T. gondii* DNA in mother's blood is may suggest a new infection or obvious clinically relevant parasitemia. The only method to detect low amounts of the *T. gondii* parasite and even damaged parasites is PCR [28]. This technique, therefore has advantages that rapid molecular methods for the diagnosis of toxoplasmosis in a clinical laboratory rather than serological methods. Several molecular methods used for detection of *T. gondii* DNA in clinical specimens either amniotic fluid or blood detection of parasitic genomic DNA. RT-PCR is used for the identification and quantification of *T. gondii* DNA in various clinical samples. The difference in test performance often concerned with a variety of factors, e.g. the structure of target genes and primers, the frequency of target replications, potential polymorphism or lack of the target sequence, and the select of the oligonucleotide sequence. The qPCR that targets repeated DNA sequences have high sensitivity for detection and quantifying of *T. gondii* in biological samples, in tissues of animal, amniotic fluids, whole blood of human clinical specimens [11]. This finding is in agreement with other studies that tiny, repetitive DNA sequence is favored of high sensitivity of RT-PCR, due to the competence of magnifying tiny DNA piece is greater than that of the huge one. Also, there are further copies of templates in a repetitive sequence in an organism. Three repetitive DNA sequences frequently utilized for the recognition of *Toxoplasma gondii* in biological specimens and clinical specimens, comprising the 35-copy B1 gene, the 300-copy 529 bp repeat part and the 110-copy internal transcribed spacer (ITS-1) or 18S rDNA gene sequences [29]. Previous researches have shown that multicopy target tests are more accurate in identifying *Toxoplasma gondii* than those that have single-copy targets [11]. The 35-repeat B1 gene, and the sequencing of AF146527, a portion of repetitive 200 to 300 times in the genome, are 2 mutual targets utilized. The ITS-1 or 18S rDNA has been utilized in a few as the marked gene in researches and demonstrated comparable sensitivity to the gene B1[30]. Comparison between RT-PCR and ELISA assays showed that 6 of 63 blood samples were found to be positive for both

RT-PCR and IgM, IgG ELISA, where one positive case RT-PCR could not be detected by ELISA against IgM and IgG. Infection diagnosis is based mainly on serological tests to assess the concentrations of anti-Toxoplasma IgM and IgG antibodies in the serum of patients. These antibodies can last for months or even years after an acute infection, but recent infections cannot be differentiated from past infections by these methods. Previous studies have reported that parasite DNA can be identified by PCR in women's blood samples before or during pregnancy. In maternal blood, the presence of Toxoplasma DNA is likely to indicate a new infection or obvious parasitemia, which perhaps to be clinically important. The simply technique that can identify low Toxoplasma gondii amounts and even damaged parasites is PCR. Many researchers recommended PCR assay to be more sensitive and specific over most serologic methods [31].

4. Conclusion

Serology is used to conduct laboratory diagnosis of toxoplasmosis, molecular techniques such as real-time Polymerase Chain reactions are also essential in confirmation of results.

References

- [1] J. P. Dubey and C. Beattie, *Toxoplasmosis of animals and man*. CRC Press, Inc., 1988.
- [2] D. E. Hill, S. Chirukandoth, and J. P. Dubey, "Biology and epidemiology of Toxoplasma gondii in man and animals," *Animal health research reviews*, vol. 6, no. 1, pp. 41-61, 2005.
- [3] B. Nagaraja, B. Ramana, D. Murty, B. Naidu, and B. Kailasanath Reddy, "Prevalence of toxoplasmosis among antenatal women with bad obstetric history," *IJPRBS*, vol. 1, no. 3, pp. 222-7, 2012.
- [4] G. Desmonts and J. Couvreur, "Toxoplasmose congénitale. Etude prospective de l'issue de la grossesse chez 542 femmes atteintes de toxoplasmose acquise en cours de gestation," *La Semaine des hôpitaux de Paris*, vol. 62, no. 20, pp. 1418-1422, 1986.
- [5] L. M. Weiss and J. P. Dubey, "Toxoplasmosis: A history of clinical observations," *International journal for parasitology*, vol. 39, no. 8, pp. 895-901, 2009.
- [6] M.-H. Lin, T.-C. Chen, T.-t. Kuo, C.-C. Tseng, and C.-P. Tseng, "Real-time PCR for quantitative detection of Toxoplasma gondii," *Journal of clinical microbiology*, vol. 38, no. 11, pp. 4121-4125, 2000.
- [7] H. T. S. A. r. Shaima Miqdad Mohamed Najeeb, Shaima mohammed Ali, "Finding the discriminative frequencies of motor electroencephalography signal using genetic algorithm," *TELKOMNIKA*, vol. 19, no. 1, pp. 285-291, 2021.
- [8] C. Contini, S. Seraceni, R. Cultrera, C. Incorvaia, A. Sebastiani, and S. Picot, "Evaluation of a Real-time PCR-based assay using the lightcycler system for detection of Toxoplasma gondii bradyzoite genes in blood specimens from patients with toxoplasmic retinochoroiditis," *International journal for parasitology*, vol. 35, no. 3, pp. 275-283, 2005.
- [9] H. T. H. H. Alrikabi, "Enhanced Data Security of Communication System using Combined Encryption and Steganography," *International Journal of Interactive Mobile Technologies*, vol. 15, no. 16, pp. 144-157, 2021.
- [10] U. Reischl, S. Bretagne, D. Krüger, P. Ernault, and J.-M. Costa, "Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes," *BMC infectious diseases*, vol. 3, no. 1, pp. 1-9, 2003.
- [11] B. Edvinsson, M. Lappalainen, and B. Evengård, "Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis," *Clinical Microbiology Infection*, vol. 12, no. 2, pp. 131-136, 2006.
- [12] N. F. A.-B. Azhar Al-zubidi, Rajaa K. Hasoun, Soukaena Hassan Hashim, Haider Th.Salim Alrikabi, "Mobile Application to Detect Covid-19 pandemic by using Classification Techniques: Proposed System," *International Journal of Interactive Mobile Technologies*, vol. 15, no. 16, pp. 34-51, 2021.
- [13] C. Pomares and J. G. Montoya, "Laboratory diagnosis of congenital toxoplasmosis," *Journal of clinical microbiology*, vol. 54, no. 10, pp. 2448-2454, 2016.
- [14] D. Ajzenberg *et al.*, "Genotype of 86 Toxoplasma gondii isolates associated with human congenital toxoplasmosis, and correlation with clinical findings," *The Journal of infectious diseases*, vol. 186, no. 5, pp. 684-689, 2002.
- [15] A. S. Abdalrada, O. H. Yahya, A. H. M. Alaidi, N. A. Hussein, H. T. Alrikabi, and T. Al-Quraishi, "A predictive model for liver disease progression based on logistic regression algorithm," *Periodicals of*

- Engineering and Natural Sciences*, Article vol. 7, no. 3, pp. 1255-1264, 2019, doi: 10.21533/pen.v7i3.667.
- [16] J.-P. Tomasi, A.-F. Schlit, and S. Stadtsbaeder, "Rapid double-sandwich enzyme-linked immunosorbent assay for detection of human immunoglobulin M anti-Toxoplasma gondii antibodies," *Journal of clinical microbiology*, vol. 24, no. 5, pp. 849-850, 1986.
- [17] M. M. Reis, M. M. Tessaro, and P. A. D'azevedo, "Toxoplasma-IgM and IgG-avidity in single samples from areas with a high infection rate can determine the risk of mother-to-child transmission," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 48, no. 2, pp. 93-98, 2006.
- [18] M. Lappalainen and K. Hedman, "Serodiagnosis of toxoplasmosis. The impact of measurement of IgG avidity," *Ann Ist Super Sanita*, vol. 40, no. 1, pp. 81-8, 2004.
- [19] A. M. Abass, O. S. Hassan, A. Rikabi, H. T. Salim, and A. Ahmed, "Potentiometric determination of fexofenadinehydrochloride drug by fabrication of liquid membrane electrodes," *Egyptian Journal of Chemistry*, vol. 64, no. 11, pp. 5-6, 2021.
- [20] A. Almogren, "Antenatal screening for Toxoplasma gondii infection at a tertiary care hospital in Riyadh, Saudi Arabia," *Annals of Saudi medicine*, vol. 31, no. 6, pp. 569-572, 2011.
- [21] R. Bahhaj, E. Ahmadpour, M. Mahami-Oskouei, E. Fallah, K. Shamsasenjan, and A. Safaiyan, "Toxoplasma gondii infection and related risk factors among blood donors in northwest Iran," *Archives of Clinical Infectious Diseases*, vol. 12, no. 2, 2017.
- [22] S. M. Bin Dajem and M. A. Almushait, "Detection of Toxoplasma gondii DNA by PCR in blood samples collected from pregnant Saudi women from the Aseer region, Saudi Arabia," *Annals of Saudi medicine*, vol. 32, no. 5, pp. 507-512, 2012.
- [23] A. Gashout *et al.*, "Molecular diagnosis of Toxoplasma gondii infection in Libya," *BMC infectious diseases*, vol. 16, no. 1, pp. 1-8, 2016.
- [24] N. Y. Doni, Z. Simsek, G. Gurses, F. Y. Zeyrek, and C. Demir, "Prevalence and associated risk factors of Toxoplasma gondii in female farmworkers of southeastern Turkey," *The Journal of Infection in Developing Countries*, vol. 9, no. 01, pp. 087-093, 2015.
- [25] P. R. Torgerson and P. Mastroiacovo, "The global burden of congenital toxoplasmosis: a systematic review," *Bulletin of the World Health Organization*, vol. 91, pp. 501-508, 2013.
- [26] Ş. Efe, Z. Kurdoğlu, and G. Korkmaz, "Seroprevalance of cytomegalovirus, rubella and toxoplasma antibodies in pregnant women of Van region," *Van Medical Journal*, vol. 16, no. 1, pp. 6-9, 2009.
- [27] I. Zednikova, E. Pazourkova, S. Lassakova, B. Vesela, and M. Korabecna, "Detection of cell-free foetal DNA fraction in female-foetus bearing pregnancies using X-chromosomal insertion/deletion polymorphisms examined by digital droplet PCR," *Scientific reports*, vol. 10, no. 1, pp. 1-9, 2020.
- [28] S. Sina, J. M. Mohammad, S. Reza, M. Anita, E. Soudabeh, and M. Hadi, "Determination of parasitic burden in the brain tissue of infected mice in acute toxoplasmosis after treatment by fluconazole combined with sulfadiazine and pyrimethamine," *European journal of medical research*, vol. 26, no. 1, pp. 1-8, 2021.
- [29] A. Coupe, "Investigating Toxoplasma gondii in the marine environment in New Zealand: from cats to kai moana (shellfish): a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Science at Massey University, School of Veterinary Science, Massey University, Palmerston North, New Zealand," Massey University, 2021.
- [30] A. F. Minutti *et al.*, "Comparison of serological and molecular techniques to detect Toxoplasma gondii in free-range chickens (Gallus gallus domesticus)," *Veterinary Parasitology*, vol. 296, p. 109515, 2021.
- [31] H. Sławska *et al.*, "Diagnostic difficulties of Toxoplasma gondii infection in pregnant women. Is it possible to explain doubts by polymerase chain reaction?," *Ginekologia polska*, vol. 76, no. 7, pp. 536-542, 2005.