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Molecular Diagnostic of Toxoplasma Gondii from Placental of Aborted Women in Mosul City

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A B S T R A C T

Toxoplasmosis is one of the most important parasitic diseases spread all over the world. It is caused by the *Toxoplasma gondii* parasite that infects mammals such as humans and animals which are intermediate hosts for the parasite. In this study, about 25 placental samples were collected from aborted women infected with Toxoplasmosis, from Al-Salam Teaching Hospital in Mosul city and microscopally examined. Serological and molecular methods were used to diagnoseding the infection in each sample .The results of this study revealed an infection rate of 100% in all placenta samples that were microscopically examined. The results of the molecular detection by convential polymerase chain reaction of the placenta samples showed that the infection rate was 48% (12 samples out of 25). Following the data from infected women, it was noted that the age group (21-30) years where the most group that were infected. The results showed a closeness of about 100% to the chain of nitrogenous bases of the isolates that were recorded during this study. The alignment analysis showed a 100% relationship with each of the Iranian isolates MK507731, the Mexican KX270373, the Egyptian OP991839 and the Saudi LN714499, and a number of isolates registered in Iraq.

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Introduction :

Toxoplasmosis is one of the most important zoonotic disease caused by an obligatory intracellular parasite called *Toxoplasma gondii* .(Sardarian *et. al.,* 2018; Gharibawi et. al., 2021).This diseases is global which has various intermediate hosts for example warm-blooded mammals, including humans, and birds (Dubey *et. al*., 2005; Pelt-Verkuil et. al., 2008), while domestic and wild cats are made as a definitive hosts for the parasite (Teweldemedhin et. al., 2019). Infection is transmitted through contaminated oocyst or tissue cyst in water or food and undercooked meat (Munoz et. al., 2011;Yan et. al., 2016). Pregnant women are infected and the infection is usually without clinical symptoms similar to influenza (Liesenfeld et al., 2001; Kadhim et. al., 2003). Parasite may be transmitted during pregnancy through the placenta from the mother to the fetus as a congenital toxoplasmosis (Tenter et. al., 2000), which causes abortion, stillbirth and severe neurological and eye disorders such as calcifications inside the brain, hydrocephalus, retinitis and blindness (Montoya et. al., 2002; Kravetz and Federman.,2005; Pappas et. al., 2009). The estimated congenital infection is 30%. It recently increases (Elise and Patrick, 2002; Yan, et al., 2016). The prevalence rate of *Toxoplasma gondii* increases with advancing age in women, and the seroprevalence rate varies in pregnant women based on the geographical area and according to the different nature, customs and traditions of countries in terms of health, social and economic aspects (Gharibawi, et al., 2021; Jones, et al., 2001).

Due to the lack of sufficient data about the local isolates of *T. gondii*, the current project is considered a detailed molecular study of toxoplasma isolates from placenta samples. This may pave the way for the development of appropriate treatment protocols for local isolates.

Materials and Methods

Sample Collection:

During this study, 25 samples of the placentas of aborted women infected with the *Toxoplasma gondii* parasite were collected from Al-Salam Teaching Hospital in the city of Mosul during the period from September to November of the same year, viz.2022. The data for each sample, such as age, number of births and number of abortions, were recorded.

1. Preparation of Samples:

Placenta samples were collected from aborted women from Al-Salam Teaching Hospital in Mosul during September 2022, (standard ethical issues were considered through the manipulation of human tissues). The samples were kept in clean and sterile containers containing phosphate buffer saline (PBS). The samples were divided into two parts: first to isolate the parasite, and the second part of the sample was placed in a neutral buffered formalin solution to preserve it until used in the molecular study (Luna,1968).

1.1 Parasite Isolation:

In order to isolate the parasite from infected placentas which were collected from aborted women previously, the placenta were cut into small pieces and suspended in 5 ml of pepsin enzyme at a temperature of 37 °C. The suspended samples were incubated at 37 °C for 10 minutes then were filtered with gauze. The mixture was centrifuged at 3000 rpm for 10 minutes. Then , the precipitate was suspended in a PBS solution with a pH of 7.2 (Dubey, 1998).

1.2 Molecular diagnostics of tissue samples:

DNA extraction from aborted placenta samples:

Parasite DNA was extracted from placental tissue samples of 25 aborted women, which showed positive results during microscopic examination in the laboratory using a special extraction kit to isolate DNA in placenta tissues. The extraction process was carried out according to the special instructions of the Korean manufacturer, Add Bio (add prepGenomic DNA Extraction kit),(Sardarian et. al., 2018).

Figure 1. Extraction of DNA of *Toxoplasma gondii* from aborted placental samples

Nested PCR for placental DNA:

The samples from which the DNA was extracted were previously prepared for genetic amplification in the polymerase chain device. DNA samples and the B1 gene of *Toxoplasma gondii*, as shown in Table 1, were prepared from the Korean manufacturer Macrogen (Homan et. al., 2000;

Sardarian et. al., 2018). The Master Mix was prepared to conduct the polymerase chain reaction by using the (Master Mix GeNetBio, Korea) kit, according to the required volumes of the reaction, and mixed in an Eppendorf tube of 0.2 ml capacity, centrifuged for 5 seconds. The volume of the master reaction was fixed at 20 microliters. After that, the PCR tubes were prepared and placed in the T 100 ™ Thermal Cycler, Bio-Rad USA, using the reaction program as shown in Table 2, inserted into a thermocycler to carry out the amplification process components 2. Finally, a separation of the PCR products was done in 2% agarose gel electrophoresis with 80 volts for 60 minutes and the results were visualized under UV light .

Sequencing and Sequence Analysis :

The PCR amplification products of B1 gene *Toxoplasma gondii* with primers (F+R) of isolated from placental were sent to Macrogen, Korea. In the NCBI GenBank . The genetic sequence of the above-mentioned gene were submitted to the GenBank to obtain the accssion number.

The Evolutionary tree.

In order to compare the isolates in the current study with the international isolates, the BioEdit program and BLAST (Basic Local Aligment Search Tool) is available on the global site of the Ncbi Genebank, which is available at the National Center for Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov)

Table 1. The nucleotide sequence of the primers used to detect the B1 gene of the parasite

No.	Temperature °C	Primer name	Primer Sequence 5' - 3'	Length
1.	58	EXB1-F	TCAAGCAGCGTATTGTCGAG	20
2.	58	EXB1-R	CCGCAGCGACTTCTATCTCT	20
3.	58	INB1-F	GGAACTGCATCCGTTCATGAG	21
4.	58	INB1-R	TCTTTAAAGCGTTCGTGGTC	20

Table 2 .Components of the Nested-PCR mix

Components	Size
Master Solution 2X Master Mix	10 µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
Distilled water without enzymes, PCR grade water	6 µl
DNA extracted from the sample (DNA Template)	2 µl
Final size	20 µl

Table 3. The steps of the PCR program to detect the B1 gene

Steps	Temperature (°C)	Duration	No. of Cycles
Denaturation DNA	95	10 minutes	1
Denaturation DNA	95	45 seconds	35
Annealing DNA	58	45 seconds	
Extension	72	1 minute	
Final Extension	72	5	1
Cooling	4	-	-

Results :

The microscopic examination of placenta samples showed that all the samples examined were 100% positive during the examination of 25 placenta samples from aborted cases.

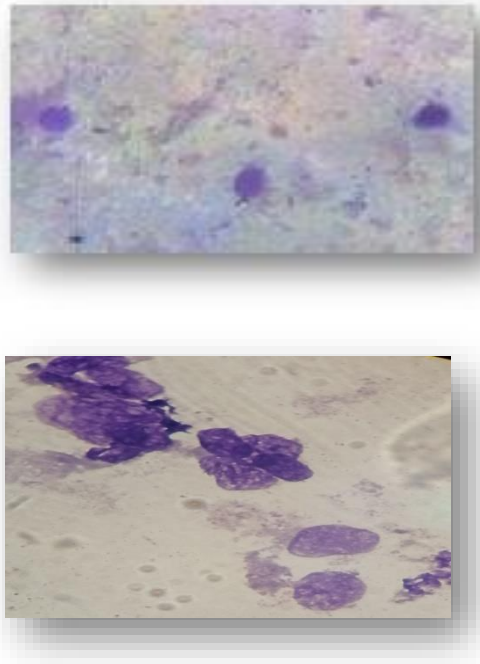

Figure 2. shows the presence of tissue cysts during microscopic examination in the tissues of the placenta of aborted women stained with Giemsa at 100x magnification.

From the following-up of the ages of the aborted women from whom the samples were isolated, the table shows that the age group of women ranging from 21-30 years were the most susceptible to infection with high rates and abortion cases were 48%.

Table 4. shows the age group of women infected with *Toxoplasma gondii* and the number of abortion .

Age (Years)	Number of Infected Women	Percentage %	Number of Abortions	Percentage %
20-10	2	8%	2	4%
30-21	12	48%	22	48%
40-31	8	32%	16	35%
50-41	3	12%	6	13%
Total	25	100%	46	100%

Molecular examination of parasite DNA isolated from aborted placenta samples:

The results of the molecular examination of the samples using Nested PCR by B1 gene *Toxoplasma gondii* , the results showed that only 48% of the samples examined during the current study gave a positive result for infection from 25 samples, as shown in Figure 3.

The results showed that the molecular interaction bands of *Toxoplasma gondii* DNA during electrophoresis had a base weight of 194 bp from *Toxoplasma gondii* DNA samples isolated from placental tissues collected during the current study.

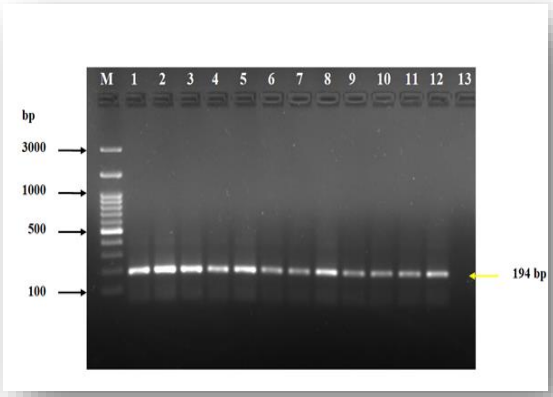

Figure 3. Agarose gel electrophoresis of the PCR product for the detection of *Toxoplasma gondii*. The path M: represents the Marker, which is 100 bp in size. The path 1 - 12: positive samples from.

Table 5. shows the percentage of infection with *Toxoplasma gondii*, using microscopic and molecular examination of placenta samples.

Sample	No.
25 The total number of placenta samples	25
The Positive sample according to microscopic examination	25 (100%)
The positive sample according to the Molecular examination	12 (48%)
The negative sample according to the Molecular examination	13(52%)

In order to identify the molecular biological aspects of the targeted B1 gene in DNA sample isolated from *Toxoplasma gondii* from aborted placenta, the genetic sequence of the parasite DNA isolated from a placenta sample was studied. The molecular sequencing of the DNA polymerase reaction product was performed.

The results showed a close relationship of about 100% to the chain of nitrogenous bases of the isolates that were recorded during the study. The results of the alignment analysis showed a 100% relationship with each of the Iranian isolates MK507731, the Mexican KX270373, the Egyptian OP991839 and the Saudi LN714499, and a number of isolates registered in Iraq in other provinces with a percentage of 100% as shown in Table (6) and Figure(5). The isolate was deposited in International Gen Bank OR020040.

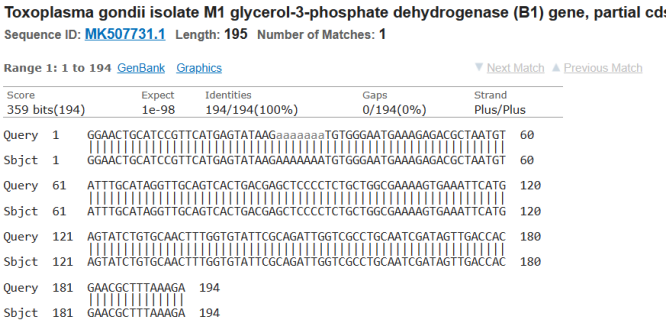

Figure 4. indicate identification of query sample, *Toxoplasma gondii*, alignment with NCBI gene bank

Table 6. Percentage distribution of parasite DNA based on partial glycerol-3-phosphate dehydrogenase (B1) gene according to nblast in GenBank of NCBI

Sample Accession Number	Bacterial Identified	Query Cover %	Identic Number %	GenBank Accession Number	Country Identification
OR020040	*Toxoplasma gondii*	100	100	MK507731	Iran
		100	100	KX270373	Mexico
		100	100	OP991839	Egypt
		100	100	LN714499	SAUDI ARABIA
		100	100	MZ717192	Iraq
		100	100	MZ717190	Iraq
		100	100	MZ717189	Iraq

100	100	MZ717188	Iraq
100	100	MZ717187	Iraq
100	100	MZ567179	Iraq
100	100	MW233648	Iraq

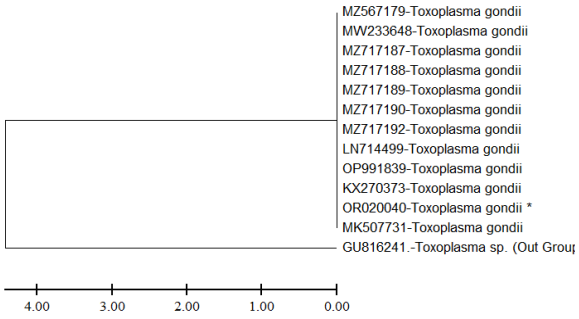

Figure5. Phylogenic tree of *Toxoplasma gondii*. The phylogenic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software and bootstrap analysis with 1000 re-samplings.

Discussion

Toxoplasmosis is one of the most common causes of abortion . The risk of infection increases during pregnancy because of its dire consequences for the mother and her fetus. The results of this study indicated that the *Toxoplasma gondii* antibodies increased at an early age in the serums of women compared with women over the age of 40 years, and agreed with the results of the study as it was noted that the majority of aborted women were of the age group (25-30) years (Abdul Mohammed *et. al.*, 2017).

Diagnosing the parasite in the placenta of aborted women using molecular diagnostic methods was important and approved in international laboratories due to its specific sensitivity in detection (ELISA and polymerase chain reaction) to diagnose the parasite in blood and tissues (Anwar and Al-bayati,2015; Montoya and emington,2008). As the results of this study showed that the age group between 21-30 years for women was the most vulnerable group to the parasite more than the younger and older than 30 years. Thus, the number of abortions at this age reached 48%. This study agreed with what was stated in (Ahmed,1992; Aryal, 2015; Teweldemedhin,2019). It was observed that seropositivity was higher in this age group, while it was lower for the younger group. It was also found that there was a relationship between age and abortion in this group. Studies have stated that the increase in the risk of abortion increases with age, and the reason is attributed to the occurrence of chromosomal changes in the egg during advancing age. It has also been shown that elderly women suffer from fertility problems (Elmore et. al., 2010). Infection in the first and second trimesters of pregnancy leads to the death of the fetus in the womb or a spontaneous abortion. While infection in the third trimester usually leads to childbirth without symptoms; yet, they appear at a later time (Ludwige.t al., 2022). This might be attributed to the lack of knowledge due to the unawareness of women's failure to following up the infection during pregnancy and conducting a serological examination to prevent toxoplasmosis. The study showed that the percentage of recurrent abortion in the first trimester of pregnancy in this stage group was higher and it coincides with what was recorded by Homan et. al.(2000); Anwar and Al-bayati (2017) who recorded an increase in the rate of IgM in the first trimester of pregnancy in Wasit Governorate. The reason for the high seroprevalence rate in this group might be due to several reasons, such as: the high rate of marriage and childbearing at this age especially in our eastern societies; also, this age represents the maximum level of activity in domestic work which gives women the greatest opportunity to be exposed to pathogens such as egg cysts and tissue cysts through cooking and cleaning as mentioned by Khan (2018) that the percentage of abortions is in the last trimester of pregnancy. Recurrent abortions in the first trimester of pregnancy may be due to a deficiency in the immune system, or the fetus may be weak and incomplete because the antibodies in the fetus are formed after the third month of birth and infection during pregnancy depends on the degree of resistance of the fetus and the immunity acquired through the placenta. The worldwide seroprevalence of toxoplasmosis varies depending on age, socioeconomic conditions, eating habits, hygiene, climate, geographic location, socioeconomic level, lack of hygiene during feeding and frequent contact with soil and cats (Mohanad et. al.,2013).

Toxoplasma gondii infections during pregnancy are associated with severe consequences for the mother and fetus, represented by abortion , stillbirth, and a number of congenital anomalies.

The high positivity of *T. gondii* in placenta samples of pregnant women from which samples were isolated during the study using the microscopy method, and confirmation of infection using the Nested PCR, is characterized by sensitivity targeting B1 with a size of 420 bp, which indicates a high incidence in women especially pregnant women.The presence of DNA does not necessarily indicate that the infection was active ,while cases diagnosed before abortion as a resulte of low immunity in this period could cause parasite activity and release of tachyzoites from tissue cysts .

This results lead to confirmation of the importance of preparing public health policies that focus on isolating and diagnosing the *Toxoplasma gondii* as

the current study recorded the first genetic isolation of *T. gondii* in pregnant women.

The close relationship of the local isolate in this study compared with the Iranian, Egyptian, and Saudi Arabian at a rate of 100% gives an indication of the accuracy of the diagnosis, and clearly puts the idea of transmission of the parasite through travel.

Conclusion

This current study revealed high infection rate of *T. gondii* in pregnant women in Mosul city. This revealed a high prevalence of toxoplasmosis in this region .

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Conflict of Interest

The researchers also declared that they have no competing interests.

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