Molecular Detection of Toxoplasmosis among Patients in Holly Karbala Province, Iraq

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Abstract

The current study was conducted in November 2023. 150 samples of malignant breast cancer tissue embedded in paraffin wax and diagnosed by a specialist doctor were collected, and 50 samples of benign tumor tissue It was used as a control group; these samples were collected from the tissue cutting laboratory in Imam Hussein Medical City, Al-Kafeel Specialized Hospital laboratory, and the specialized AL-Sajad laboratory for histological and cellular examinations and tumor diagnosis in Holy Karbala. The study samples included two groups. The first group consisted of 66 women with breast cancer who did not undergo chemotherapy; the second group was 84 women with breast cancer who underwent chemotherapy. PCR technology was performed on DNA in tissue samples affected by breast cancer for all groups under study, based on the amplification of the B1, SAG1, gene; the current study aimed to determine the incidence of toxoplasmosis in breast cancer patients. The incidence of toxoplasmosis in breast cancer patients was 11.3%, and the infection rate in the healthy group was 4%. The results showed that the infection rate in women who received treatment was higher than in women who did not receive treatment due to weakness, their immunity, and the risk of infection, increasing the probability of sick women developing breast cancer by 3.7% compared to healthy women.

Keywords: Breast cancer, FFPE, tissues, PCR, *T.gondii*, women, Genotype.

Introduction

Toxoplasmosis is a zoonotic illness caused by the parasite Toxoplasma gondii. This obligate intracellular protozoan infects humans and warm-blooded animals as intermediate hosts, while various members of the feline family serve as final and intermediate hosts for the parasite. The parasite infects people by ingesting food or water polluted with oocysts produced in the feces of infected cats or by consuming raw or inadequately cooked meat harboring tissue cysts. It may also be transferred congenitally from an infected mother to her fetus (1), (16). Toxoplasmosis infection is ubiquitous among people, varying by geography, with around one-third of the global population subject to the parasite. The infection is usually asymptomatic in immunocompetent individuals, while the infection is severe and dangerous in immunocompromised people, for example, pregnant women and infected people with AIDS (3). The parasite is transmitted from the infected pregnant mother to the fetus through the placenta. It causes congenital malformations and miscarriages, mainly if the infection occurs during the first trimesters of pregnancy (17). However, suppose the infection occurs during the third trimester of pregnancy. In that case, the infection may not cause Clinical signs in children in the early stages of life. However, as the child ages, other signs may develop, for example, retinitis, chorioretinitis, or ocular toxoplasmosis. The severity of the disease is determined by a few factors, including host immunity, parasite strain, the size of the infectious dose, and the type of immune response for the host (4). Cancer is among the primary causes of mortality globally (5). Notwithstanding the precise identification of this malignant neoplasm, it remains the predominant cause of cancer-related mortality (6). Over the last twenty years, there has been a notable rise in breast cancer incidence (7), which is the second most frequent cancer kind behind lung cancer and the most widespread malignant illness affecting women globally (8). Research indicates a possible correlation between toxoplasmosis and malignant conditions, such as brain and mouth malignancies (9). Moreover, in Iran, the DNA of the parasite *T. gondii* was

detected in breast cancer tissue fixed with formalin and paraffin wax (10). Some studies have indicated the possibility of toxoplasmosis being linked to other types of cancer, such as leukemia, ovarian, and lung cancer; one study also indicated that the Toxoplasma gondii parasite can cause tissue damage in the reproductive system and abnormal adhesions inside the uterus, causing infertility in women. (12), (11). The diagnosis and genetic characterization of the Toxoplasma gondii parasite are crucial for clinical care and epidemiological research, as well as for controlling the parasite in people and animals.

Interaction (12) indicates that Polymerase Chain Reaction (PCR) has been extensively utilized for the detection of Toxoplasmosis infection since its first application by Burg et al. (1989) to target the B gene (13). Traditional polymerase chain reaction targets single-copy genes in humans and animals, including SAG1, SAG2, SAG3, and GRA1 (14).

Materials and methods

150 samples of malignant breast cancer tissue embedded in paraffin wax and diagnosed by a specialist doctor were collected, and 50 samples of benign tumor tissue were used as a control group. These samples were collected from the tissue sectioning laboratory in Imam Hussein, the Medical Laboratory, the Al-Kafeel Specialized Hospital Laboratory, and the Al-Sajjad Specialized Laboratory for histological and cellular examinations and tumor diagnosis in Holy Karbala. The study samples included two groups. The first group was 66 women with breast cancer who did not undergo chemotherapy. The second group was 84 women with breast cancer who underwent chemotherapy. PCR technology was performed on deoxyribonucleic acid (DNA) in tissue samples affected by breast cancer for all groups under study, based on gene amplification. B1 and SAG1.

Deoxyribonucleic acid was extracted from breast cancer tissue samples. Five 10-micron thick sections were cut from breast tumor tissue embedded in paraffin wax to isolate the DNA genome using the Genomic DNA Mini Kit Tissues Protocol, which was designed specifically for this purpose. The Korean commercial kit, produced by Favorgen, was used to extract DNA from breast cancer tissue samples.

Method work:

First: Sample Preparation & Tissue disassembly

The tissue sections, after being trimmed of excess wax, were transferred to 1.5 ml Eppendorf tubes. 1 ml of xylene was added to the sample; then the mixture was mixed with a mixer and incubated for 30 min at room temperature, then placed in the centrifuge at maximum speed for five minutes; it has been withdrawing the supernatant liquid using a micropipette. 1 ml of absolute ethanol was added to wash the sample with continuous mixing. The tubes were put in the centrifuge at maximum speed for 5 min, ignoring the liquid. The caps of the Eppendorf tubes were left open and incubated at 37°C for 15 min to remove the remaining ethanol alcohol and evaporate it. The tissue was crushed using a pestle, and then 200 microliters of FATG1 buffer were added to the sample. Mixing was continued using the pestle.

Second: Lysis

Twenty microliters of Proteinase K were introduced to the sample and stirred using a mixer for several seconds. Subsequently, the specimens were incubated at 60°C for 1 hr, stirring every 10 minutes for analysis. 200µl of buffer was added from FATG2. The sample was mixed well with a mixer for 5 seconds, then incubated at 70°C for 10 minutes. The samples were shaken every 5 minutes to decompose the tissue well. The centrifugation was repeated at maximum speed for two minutes in case there were undissolved materials after incubation. The centrifugation was then transferred to a centrifugation tube. New 1.5ml.

Third: DNA binding

200 microliters of 100% ethanol were added to the specimen and thoroughly blended with a mixer for 10 sec, after which only the liquid was extracted. The eluent was transported to the filter tube's column after being positioned in a 1.5 ml collecting tube. The tubes underwent centrifugation at maximum velocity for two minutes. The filtrate was disposed of in the collection tube, and the filtration tube was relocated to a new collecting tube.

Fourth: Washing

400 µl of WI buffer was added to each GD column and centrifuged at max speed for 1 min. The filtrate was then transferred to the collecting tubes, and the GD column was returned to the collecting tubes. Six hundred microliters of Wash Buffer combined with ethanol were applied to the filter tube. The tubes were ejected at max velocity for one minute, after which the filtrate was transferred into the collecting tube. This process was repeated at the same speed for 3 min to dry the filter tube material.

Fifth: Elution

The dry filter tube was relocated to a fresh collection tube, and 100 microliters of DNA elution buffer were introduced. The filter tube was allowed to absorb the elution buffer for 5 min, followed by centrifugation at max speed for 2 min. After that, the deoxyribonucleic acid precipitate was taken and stored at -20 degrees Celsius.

The DNA extracted from all the tissue samples was detected using a NanoDrop Spectrophotometer device produced by the Taiwanese company ACTGENE. It is used to detect nucleic acids (DNA and RNA) by measuring the nucleic acid concentration in nanograms per microliter and reading the absorbance intensity and wavelength. 280/260nm, In addition to measuring the purity of DNA, DNA is considered pure when its absorbance intensity is at a rate of (1.8±0.2) (2004, Melville), noting that the average concentration of DNA extracted from the study samples is 180 nanograms/microliter.

The polymerase chain reaction was prepared using a commercial PCR premix 20µ Reaction kit by the Korean company Addbio. Bio-Tech and according to the company's instructions (Table 1)

Table 1. Master Mix components for the polymerase chain reaction

Components	**Volume** (µL)
Polymerase enzyme Taq DNA Polymerase	1U
Each: d NTP(d ATP, CTP,d GTB,dTTP A mixture of nitrogenous bases	400 µL
Buffer solution	10 µL
Loading dye	30 µL
MgCl2	3 µL

The primer used in PCR Reaction.

Specific primers were used for the B1 gene based on (18) and the SAG1 gene based on (19). It is equipped by the Korean company Bioneer, and according to Table 2:

primers sequence (5`-3`)			PCR product size
B1Gene	Primer Forward	GGAACTGCATCCGTTCATGAG	194bp
	Primer Reverse	TCTTTAAAGCGTTCGTGGTC	
SAG1Gene	Primer Forward	TTGCCGCGCCCACACTG ATG	241bp
	Primer Reverse	CGCGACAC AAGCTGCGATAG	

Determination of Toxoplasma genotype

The genotype of the five nuclear samples extracted from malignant breast cancer tissue for 17 samples positive for Toxoplasma gondii was determined if the SAG1 gene was used according to the primers used in Table 2, which was done according to the information provided by the manufacturing company that used the "Add Prep Genomic DNA Extraction Kit." (Addpio®, South Korea) As previously described and according to the thermal cycling program in Table (5), these primers were used in DNA sequence analysis. This approach was carried out using special cutting enzymes according to methods outlined by (20)

Preparing the polymerase chain reaction (PCR) Mixture

This mixture was produced based on the manufacturer's instructions in Table 3. All components mentioned in the Table were placed in special tubes. The tubes were closed and transferred to the microcentrifuge at maximum speed for half a minute. The tubes were transferred to the PCR thermal cycler.

PCR PCR Reaction Mixture	(µL) **Volume**
DNA DNA Extraction	2
Primer Forward	2
Reverse Primer	2
Free DNAase water	6.5
Mastermix	12.5
Total volume	25

Table(3) PCR Reaction Mixture

PCR Program PCR Thermo cycle

The polymerase chain reaction was performed utilizing a thermal cycler for the PCR reaction produced by the Chinese company Biobase, as the target gene SAG1 and B1 were amplified to a size of 194bp and 241bp, respectively, as shown in Tables (4) and (5):

(Table 4): The program used for the B1 gene in the PCR machine

Step	**Temperature (Cº)**	**Time**	**Cycle's Number**
DNA Initial Denaturation	95	3min	35
Denaturation	95	3s	35
Annealing	60	30min	35
Extension	72	1	35
Final extension	72	5	35

(Table 5): The program used for the SAG1 gene in the PCR machine

Step	**Temperature (Cº)**	**Time**	**Cycle's Number**
DNA Initial Denaturation	95	3min	35
Denaturation	95	3s	35
Annealing	63	30min	35
Extension	72	1	35
Final extension	72	5	35

Electrophoresis of PCR Product by Agarose Gel

Electrophoresis was performed using agarose gel to determine the result of the polymerase chain

reaction. I placed 5 microliters of the DNA Ladder (100-2000) in the first hole. Then, after completing the samples and measuring ladder placement, the agarose gel was immersed in the TBE Buffer solution, the concentration of which was 10 ml of the buffer solution with 100 ml of distilled water. The fluorescent ethidium bromide dye was used in Red. An electric current with a voltage difference of 60 volts was passed for half an hour. Then, after the end of the relay period, the device was disconnected from the current and electrophoresis, and the template was lifted and transferred to the EZ-capture-MG device to be examined using a U.V light source to determine the result with the measurement unit; the result is considered positive by observing bands of DNA from the B1 gene, with a size of 194 bp, and for the SAG1 gene, with a size of 241 bp, and comparing the results with the measurement scale. The ladder and the samples were photographed using a digital camera attached to an EZ-capture MG device.

Statistical analysis

As applicable, categorical variables are represented as numbers and percentages (%) and assessed through chi-square tests or Fisher's exact test. Continuous variables are presented as mean ± standard deviation (SD) and analyzed using Student's t-test. Risk factors for breast cancer were assessed utilizing a logistic regression model and presented as odds ratios (OR) with associated 95% confidence intervals (CI). All analyses were conducted with SPSS version 28. Variations with p-values less than 0.05 were deemed statistically significant.

Results and discussion

Table 6. Study groups according to infection.

	Study groups				**Total**		X^2 **p-value**	**OR (95%CI)**	**RR (95%CI)**
***Toxoplasma*. test**	**patients**		**Control**						
	N	**%**	**N**	**%**	**N**	**%**			
Ve+Toxo.	17	11.3%	2	4.0%	19	9.5%	**2.346 0.126 Ns**	**3.07 (0.68-3.77)**	**2.83 (0.68-11.84)**
Ve- Toxo.	133	88.7%	48	96.0%	181	90.5%		**Ref.**	

This research identified 150 women with breast cancer, with an average patient age of 25 to 56 years. Seventeen women infected with Toxoplasma gondii were documented, as shown in Table 6:

Table 7. The relationship of *Toxoplasma gondii* with age groups in patients

Study groups		**patients**		**Control**		**p-value**
	# Mean ± SD	**No.**	**50.77 ±11.09**	**No.**	**44.16 ±13.80**	**0.003***
Age *groups*	**25-34 year**	8	5.3%	15	30.0%	X^2**= 22.861**
	35-44 year	33	22.0%	10	20.0%	**<0.001***
	45-54 year	53	35.3%	13	26.0%	
	> 54 year	56	37.3%	12	24.0%	

The age groups most infected with Toxoplasma gondii were 45-54, as it was detected by polymerase chain reaction using the B1 gene and the SAG1 gene. These samples included breast tissue embedded in paraffin wax infected with cancer. 50 samples of benign breast tissue were examined as a control group, and the results showed two samples positive for Toxoplasma gondii infection. These results were similar to those of a study conducted in Kirkuk. The study found that the highest rate of toxoplasmosis infection was recorded among women with breast cancer within the age group of 50-59 years, while the lowest rate was in the age group of 20-29 years, and the rate

increases significantly with increasing age of the patients, (21). The reason for this is attributed to the weakness of the immune system, the incidence of chronic diseases, and the nature of foodstuffs, which are possible reasons that increase *Toxoplasma gondii*.

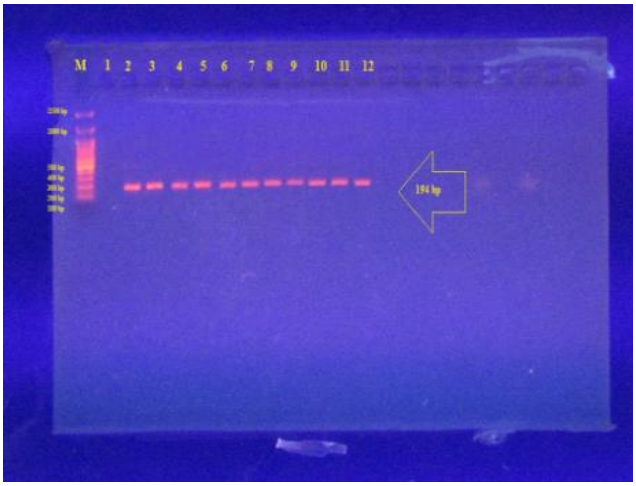
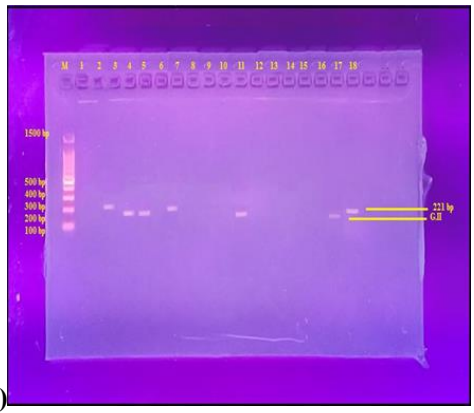
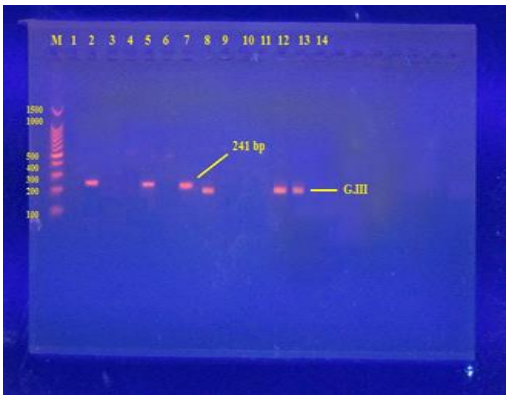


Figure 1: Detection of the DNA of B1 gene

Electrophoresis of the polymerase chain reaction (PCR) product) of the *Toxoplasma gondii* parasite(B1 gene) on an agarose gel with a voltage of 60 for half an hour and using the fluorescent red dye Ethidium Bromide, as the samples show a band with the size of 194 nitrogenous base pairs paths DNA isolated from tissues of breast cancer patients.

(A)





(B)

Figure 2: (A) Detection of the DNA of SAG1 gene type.II, (B)Type. III

Electrophoresis of the polymerase chain reaction (PCR product)) of the gene of the Toxoplasma gondii parasite (gene SAG1 *Toxoplasma gondii*) on an agarose gel at a voltage of 60 for half an hour and using the red fluorescent dye Ethidium Bromide, as the samples show a band the size of Sau3AI enzyme for G. III 241 bp nitrogenous base pair and HhaI enzyme for G.II product 221 as the paths show isolated DNA from tissues of breast cancer patients.

Table (8): *Toxoplasma gondii* genotype in cancer patients

Genotype	Study groups				Total		X^2 p-value	OR (95%CI)	RR (95%CI)
	patients		Control						
	N	%	N	%	N	%			
Type II	4	23.5%	0	0.0%	4	21.1%		**Ref.**	
Type III	13	76.5%	2	100.0%	15	78.9%	**8.07**		**1.15 (0.95-1.41)**

							0.005*		
X^2 **p-value**	**4.77 0.03***		-						

T. gondii has three main genotypes, I, II, and III, with recombined and atypical strains. PCR-based molecular genotyping was used in tissue samples; in the present study, the SAG1 marker was used to determine the genotype of the isolates. Genotype III was more prevalent in women with breast cancer, and genotype II agreed with a test study in any study due to the use of Mn-PCR-RFLP in the current study, which is more visible and accurate than the traditional polymerase chain reaction (20).

Table 9: Effect of chemotherapy on *Toxoplasma gondii* patients

Effect of chemotherapy	***Toxoplasma gondii***				**Total**		X^2 **p-value**	**OR (95%CI)**
	Ve+		**Ve-**					
	N	%	N	%	N	%		
After of chemotherapy	13	76.5%	66	49.6%	79	52.7%	4.358 0.037*	3.30 (1.02-10.64)
Before of chemotherapy	4	23.5%	67	50.4%	71	47.3%		Ref.
Total	17	100.0%	133	100.0%	150	100.0%		

The current study showed the effect of chemotherapy on *Toxoplasma gondii* infection, and it was noted that there was a difference between the number of infected women who received treatment and those infected who did not. The findings were similar to research performed in Egypt assessing the incidence of Toxoplasma gondii among cancer patients [22]. This results from the heightened number of chemotherapy stages (utilizing immunosuppressive agents), which compromise patients' immune systems and diminish their defenses against infections. Malignant illnesses are linked to a deficiency in cell-mediated immunity, referred to as the T-cell exhaustion hypothesis, exacerbated by using immunosuppressive medications. This results in the onset of toxoplasmosis and the immune system's failure to manage infections caused by intracellular microorganisms and malignancies.[23,24]

The rise in immune system problems and the utilization of immunosuppressive medications has underscored the significance of toxoplasmosis as an opportunistic infection that may result in mortality. Notwithstanding its crucial significance, toxoplasmosis has not garnered the attention it deserves. Toxoplasmosis now poses a significant hazard to public health. Most research examining the correlation between cancer and Toxoplasma gondii infection depends on assessing the incidence of the parasite in serum among cancer patients rather than doing direct tissue sample analyses. In all, 8.38% of screened patients with malignant tumors in China tested positive for Toxoplasma gondii antibodies. Nevertheless, when nested PCR detection was applied to identical specimens, just 3.55% of these individuals tested positive [27]. Separate research conducted in China revealed a far greater frequency, with 35.56% of cancer patients testing positive for anti-T. gondii IgG. This research reported the most significant frequency of infection [28]. Lung cancer patients constituted 60.94%, followed by cervical cancer patients at 50%. In a cohort of 356 cancer patients, 21 (5.9%) tested positive for IgG, 8 (2.3%) for IgM, and five exhibited both IgG and IgM antibodies [29]. The total prevalence of Toxoplasma infection in this research was 6.8% [29]. A study in Iran revealed that 45.2% of cancer patients tested positive for T. gondii serum [23]. Elevated seropositivity rates have been seen in women diagnosed with breast cancer, at 86.4% [24]. Research comparing national data from 37 countries revealed that brain tumors are 1.8 times more prevalent in nations with a high incidence of T. gondii infection compared with those with little presence of the parasite. Numerous studies have shown a correlation between Toxoplasma gondii infection and numerous kinds

of cancer [32, 33, 34, 35]. According to one study, 46.2o% of women with breast cancer and reproductive system infections showed antibody test results for T. gondii. Positive, and 93.7% of these women had antibody levels of 1:400 or higher[36]. After leukemia and lymphoma, a recent study found a higher serum prevalence of toxoplasmosis in breast cancer patients [35].

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