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Single Nucleotide Gene Polymorphism of Toll-Like Receptor 2 to Toxoplasmosis in Recurrent Aborted Women

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Abstract

Background: This study was conducted at the College of Medicine – Al-Qadisiyah University in Cooperation with the women and children in Wasit hospitals from the period January to October -2022. It has been carried out on 102 blood samples from aborted women and congenital anomalies mothers referred to the hospitals 50 blood samples from pregnant women as control cases, and 50 blood samples as a control group obtained from healthy pregnant women. The objective was to determine the effect of *TLR2* gene polymorphism on seropositive toxoplasmosis in aborted women

Methods: The research aimed to explore how the (TLR2) gene polymorphism influences the occurrence of miscarriages in women. This was investigated using the ARMS-PCR technique, which utilized allele primers to identify Toxoplasma gondii infection in women who experienced clinical miscarriages. The study also assessed the genotypes for the TLR2 G/A (rs1898830) SNPs through ARMS PCR assays, and all genotypes were evaluated for compliance with Hardy-Weinberg (H-W) equilibrium.

Results: All the analyzed genotypes preserved the H-W equilibrium among the studied groups of patients (P >0.942 and 0.893). The result of gene polymorphism of (TLR2, SNP rs-1898830) in Toxoplasma aborted women, showed that the homozygote mutant AA appeared 19, and heterozygote mutant GA appeared in 53was a risk factor for abortion in TLR2, and the difference was non-significant.

Conclusion: The current study concluded that genetic polymorphisms of TLR-2 had a risk factor role in toxoplasma patients, which many correlated with aborted women, rather than pregnant women.

Introduction

T. gondii is widespread worldwide, although it varies from place to place In the world [1]. *Toxoplasma* protozoa is an obligatory intracellular protozoan that can infect all nucleated cells [2]. It is capable of producing severe symptoms and causing the parasite to multiply in a life-threatening way in pregnant women and immunocompromised persons [3]. People are often infected by the eating of raw or undercooked meat with tissue cysts, or through water and food contaminated with Oocysts found in cat feces [4]. Infections are passed directly from the mother to the fetus during the active phase of tachyzoite transition through the placenta, rarely via blood transfusions or organ transplants [5]. Congenital infection is one of the most serious complications of toxoplasmosis in pregnant women. Congenital transmission of Toxoplasma gondii occurs most often during pregnancy [6]. Toll-like receptors (TLRs) are naturally encoded immune sensors that are present from birth and have the ability to identify common microbial patterns and signals from the host's own tissues. They trigger the production of major histocompatibility complex (MHC) proteins, molecules that aid in co-stimulation, and inflammatory agents in various cell types like macrophages, neutrophils, dendritic cells, and others. These activities set into motion immediate and early mechanisms of the innate immune system, while also kickstarting and coordinating adaptive immune responses [7]. Among TLR characteristics for human cells, the activity in sensing *T. gondii* was especially determined for TLR2, TLR4, and TLR9 molecules [8] Numerous molecular diagnostic methods were used for *T. gondii* identification such as conventional tests, ARMS–PCR, and real-time-PCR. ARMS-PCR is one of the most accurate tools in genetic disease diagnosis in recent days [9-11]

Methods

The study included 102 aborted women and moms with congenital defects who have had multiple abortions, as well as fifty healthy women who had a normal birth. From January to September 2022, samples were collected from suspected patients and a control group at AL-Kut for Women and Children Hospital in Wasit Province. They ranged in age from 15 to 45 years.

Blood Samples

Using disposable syringes, two milliliters of venous blood were extracted from the veins of each suspected patient and control group. Two milliliters of this blood were collected in an EDTA tube by micropipette and kept at -20 °C until analysis (DNA extraction).

The Diagnostic Methods:

Serological test

The serological test was performed to diagnose the parasite Toxoplasma gondii, and an ELISA IgG kit with the catalog number EIA-3519 was utilized.

Molecular detection test

Amplification Refractory Mutation System Polymerase chain reaction (ARMS-PCR**)**. Blood Protocol Genomic DNA Kit was used following the DNA extraction kit. With Geneaid Biotech Ltd, the ARMS-PCR and Gel electrophoresis procedures were in accordance [12].

Primers

This work used the NCBI-SNP data source and Primer1 Allele Specific-PCR primers designed online to create allele-specific-PCR primers for TLR2 gene polymorphism. These primers were given by (Scientific Research Co. Ltd. Iraq) and are listed in table (1).

Genomic DNA Extraction (blood)

The genomic DNA was obtained from blood samples using the Geneaid gSYAN DNA extraction kit designed for frozen blood samples, and the procedure was as follows: 200 microliters of frozen blood were placed into a sterile 1.5 ml microcentrifuge tube. Afterward, 30 microliters of proteinase K were added, and the mixture was thoroughly mixed using a vortex. It was then incubated at 60°C for 5 minutes. DNA extraction involved several sequential steps. First, each tube received 200 microliters of lysis buffer GSB, and thorough mixing was achieved using a vortex. The tubes were subsequently incubated at 70°C for 10 minutes, with periodic inversion every 3 minutes throughout this incubation period. Following this, 200 microliters of 100%ethanol added to the lysate and vigorously mixed. Next, DNA filter column was positioned inside a 2 ml collection tube, and the entire mixture, including any precipitate, was transferred to the column. The mixture underwent centrifugation at 10,000 rpm for 5 minutes, with the 2 ml collection tube used to collect the flow-through. Then, the column was transferred to a fresh 2 ml collection tube. Subsequently, 400 microliters of W1 buffer were introduced into the DNA filter column, which was then centrifuged for30s at 10,000 rpm. The flowthrough was discarded, and the column was reinserted into the 2 ml collecting tube. Each column was treated with 600 microliters of Wash Buffer containing ethanol. To facilitate drying of the column matrix, all tubes were centrifuged for3 min. at 10,000 rpm. Finally, the dried DNA filter column was placed into a clean 1.5 ml and 50 microliters of preheated elution buffer were added to the center of the columnmatrix. After allowing the tubes to stand for at least 5 minutes to allow absorption of the elution buffer

by the matrix, the purified DNA was centrifuged for 30 seconds at 10,000 rpm.

Allele specific-PCR Method

Allele specific-PCR assay was TLR2 gene polymorphism was detected and genotyped in blood samples from aborted patients and healthy controls. This approach was carried out following (15).

Allele Specific-PCR Master Mix Preparation

Allele Specific-PCR master mix was created using the (GoTaq® G2 Green Master Mix kit), and this master mix performed two reactions for each sample according to the manufacturer's instructions.

Allele Specific-PCR Thermocycler Conditions

Denaturation steps in the PCR thermocycler were as follows: initial denaturation 95°C 5 min. 1 (repeat), denaturation 95°C 30 sec. 35 cycle, Annealing 58°C 30 sec. 35 cycle, Extension 72°C 30 sec. 35 cycle, and final extension 72°C 5 min. 1 (repeat).

Statistical Analysis

SPSS (version 25) was used for statistical analysis of the data. By comparing observed and anticipated frequencies, the polymorphisms were assessed for divergence from Hardy Weinberg Equilibrium (HWE). The link between genotype and risk of toxoplasmosis was assessed using logistic regression analysis and the Odds ratio (OR) with 95% confidence interval (95%CI). The statistical significance level was chosen at 0.05 (16).

Ethical consideration

The ethics of scientific research was not only the personal traits of the scientific researcher, but it was the real and organized responsibility of the scientific researcher and his academic research, and it is an integrated system that clarifies the rights and duties and organizes research institutions and associations where the patient's consent was obtained to allow samples to be taken, as well as the approval of the Al kut Hospital for Women and Children from December -2021

Results

The positive results were seen in 27 (28.7 %) of the aborted women in comparison with 67 (71.3 %) of the aborted women who were negative, and all the patients with congenital anomalies 8 (100.0 %) had positive results of IgG. while all the healthy controlled subjects 50 (100.0 %) had negative results of IgG, and the variation was significant, (P= 0.001) in table (2). The study showed that the ages between 20-29 years were more affected by aborted women, where the incidence rate reached 48% in Table (3). The distribution of *TLR2 (rs1898830)*Polymorphism was detected by the ARMS-PCR technique. In Figure (1), the wild-type homozygote genotype showed only the G allele, the mutant-type homozygote genotype showed only the A allele, and the heterozygote genotype exhibited T and A alleles. The genotype distribution had no deviation from Hardy-Weinberg equilibrium. The association between the risk of disease and TLR2 (rs*1898830*) gene polymorphism is shown in Table (4).The relationship between *TLR2 (rs1898830) POLY* gene polymorphism and the risk of illness displays. The homozygous genotype AA was more numerous in the (PG) compared to the (CG) (18 vs. 7, respectively), although the difference was not statistically significant (P = 0.733). In the light of this, genotype AA was a risk factor for abortion. Despite this, the heterozygous genotype GA was more common in the (PG) than in the (CG), with 34 vs. 17 cases, respectively, and the difference was not statistically significant (P = 0.733). In light of this, genotype GA was found to be a risk factor for abortion. Table (5) shows the association between TLR2 (rs1898830) allele polymorphism and illness risk.

Primer	Sequence (5'-3')	Product size
Wildtype Reverse Primer	TAGTAAAATAAATCCAGAGAAATCG	537bp
Mutant Reverse Primer	TAGTAAAATAAATCCAGAGAAATCA	
Common Forward Primer	AGCATCCAACAGTGTGAGCA	

Table 1: Primers used in this study

IgG result	Aborted Women	Congenital anomalies	Control	P value
Positive, *n* (%)	27 (28.7 %)	8 (100.0 %)	0 (0 %)	P= 0.001 ¥ S
Negative, *n* (%)	67 (71.3 %)	0 (0%)	50 (100.0%)	
Total	94 (100.0%)	8(100.0%)	50 (100.0%)	

¥: Chi-square test; *n*: number of cases; **S**: significant at *P*< 0.05

Table 2: Serum IgG results in the detection of *T. gondii* infection in aborted women, congenital anomalies, and healthy control.

Age (years)	Characteristic		
	Positive IgG results	Negative IgG results	
< 20 years, *n* (%)	2 (7.4 %)	4 (6.0 %)	0.894 ¥ NS
20-29 years, *n* (%)	13 (48.2%)	38 (56.7%)	
30-39 years, *n* (%)	9 (33.3%)	18 (26.9%)	
⩾ 40 years, *n* (%)	3 (11.1 %)	7 (10.4 %)	
Total	27	67	

¥: Chi-square test; *n*: number of cases; NS: not significant at *P*> 0.05.

Table 3: Frequency distribution of IgG Elisa results depending on age groups.

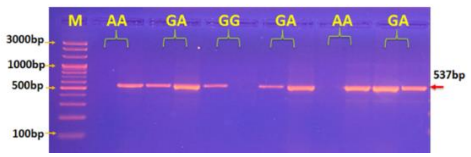

Figure 1: Image from an agarose gel electrophoresis showing the detection of the TLR2 rs1898830 G/A gene polymorphism by ARMS-PCR. Where M: marker (2000-100bp). Just the G allele was shown in the lane (GG) wild-type homozygote. The homozygote mutant shown in lane (AA) was solely carrying the A allele, while both G and A alleles were present in the (G/A) heterozygote. G or A alleles were found in the 537 bp product size.

TLR2 (rs1898830)	Aborted women	Congenital anomalies	Control	P1
AA	18	1	7	0.942
GA	31	3	17	¥
GG	45	4	26	NS
Total	94	8	50	
TLR2 (rs1898830)	P2			
Ab vs GA	0.893			
Ab vs C	0.733			
GA vs C	0.980			

P1: overall comparison; P2: Individual genotype comparison versus reference; n: number of cases; ¥: Chi-square test; Ab: Abortion patients; GA: Congenital aborted C: Control: NS: Not significant at P < 0.05.

Table 4: Genotypes frequency of *TLR2 (rs1898830)* POLY in patients and control group

TLR2 (rs1898830)	Abortion patients	Congenital anomalies	Control	P1
A	67	5	31	0.607
G	121	11	69	¥
Total allele	188	16	100	NS
TLR2 (rs1898830)	Statistic			
	P2	OR	95%CI	
Ab vs GA	0.724	1.218	0.404-3.651	
Ab vs C	0.429	1.232	0.734-2.07	
GA vs C	0.984	1.012	0.323-3.16	

P1: overall comparison; P2: Individual genotype comparison versus reference; n: number of cases; ¥: Chi-square test; OR: odds ratio; CI: confidence Interval

Table 5: Alleles frequency of *TLR2 (rs1898830)* inpatients and control group.

Discussion

Serological methods for detecting anti-toxoplasma antibodies in the body after exposure to infection are considered one of the very important methods, as the body produces IgM immune antibodies when Primary infection, which is characterized by a short duration of its presence [13]. Whereas the emergence of IgG antibodies after a period of initial infection provides a highly efficient and approved diagnostic tool for the detection of previous exposure to Toxoplasma infection, which lasts a lifetime [14]. In this study, the results agree with, Darweesh and Sarker [15,16]. The seroprevalence of toxoplasmosis shows a widespread distribution all over the world depending on age, geographical regions, dietary habits, immune status, hygienic status, sanitary supplies, and socioeconomic status [17]. The study showed that the ages between 20 and 29 years are more affected by aborted women, where the incidence rate reached 48%, and this study is consistent with, Hamza and Kheirandish [18,19]. This study used ARMS-PCR to evaluate the relationship between particular SNPS in the *TLR-2* rs-*1898830* genes and recurrently aborted women. This study was consistent with Wujcika et al., [20], this previous study showed that H-W equilibrium was preserved. This study was consistent with Wujcika et al., [21] and Taniguchi et al., [22], Nevertheless, this earlier study found that infants with congenital CMV infection tended to have a reduced frequency of the GA heterozygous genotype at the rs1898830 gene in TLR-2, In other words, children with congenital CMV infection had a greater prevalence of the homozygous AA genotype than the overall population.

The current study concluded that genetic polymorphisms of TLR-2 had a risk factor role in toxoplasma patients, which many correlated with aborted women, rather than pregnant women.

Author Contributions

Conceptualization : Mohammed Hassan Aubed,Ghada Basil Ali Alomashi:
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Funding Acquisition: Mohammed Hassan Aubed,Ghada Basil Ali Alomashi:
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Competing Interests

The authors declare that there is no conflict of interest.

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