

Molecular Detection of *Toxoplasma gondii* via SAG 2 gene in thalassemic patients using nested polymerase chain reaction analysis

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Abstract

Toxoplasma gondii infections are prevalent in humans and animals. This study aimed to detection SAG2 gene by nested PCR method in Iraqi thalassemic patients infected with *T. gondii*. Patients sample consist of 165 were collected from Al- Karma Teaching Hospital in Baghdad, Iraq, also 80 case of healthy individuals their age ranges from 2-45 years, with mean (15.387±0.627) during the period from March to June 2022 cases Iraq. Anti-*Toxoplasma* IgM/IgG antibodies were using in detection toxoplasmosis by Chemiluminescent microparticles immunoassay (CMIA). Results revealed that there is a significant difference $P \leq 0.001$ in the level of *Toxoplasma* IgG antibody between group of negative control group (0.5616±0.246 IU/ml) with β -thalassemic major patients infected with toxoplasmosis (24.985±6.101 IU/ml) and positive control infected with toxoplasmosis (35.59±8.336 IU/ml), also the difference was significant ($P < 0.001$) between the group of positive control infected with toxoplasmosis (35.59±8.336 IU/ml) with the group of with β -thalassemic major patients (0.489±0.084 IU/ml), however all groups were seronegative for anti-*Toxoplasma* IgM antibody. Molecular detection was done by nested PCR method to detect SAG2 gene in studied samples. Results showed that 6/25(24.0%) of β -thalassemic major patients infected with toxoplasmosis have positive response SAG2 gene by nested PCR assay, while others groups were negative for SAG2 in the same assay. In conclusion, the Iraqi B-thalassemia patients of the current study were infected with chronic toxoplasmosis. Furthermore, result showed present of *T. gondii* SAG2 gene were detected in the blood samples of B-thalassemia patients with toxoplasmosis and positive control.

Keywords: *Toxoplasma gondii*, Thalassemia, SAG2 gene, Nested PCR

الكشف الجزيئي عن طفيلي المقوسة الكوندية عبر جين SAG 2 لدى مرضى التلاسيميا باستخدام تحليل تفاعل البوليميراز المتسلسل المتداخل
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المستخلص

تنتشر عدوى المقوسة الكوندية لدى البشر والحيوانات. هدفت هذه الدراسة إلى الكشف عن جين SAG2 باستخدام تقنية تفاعل البوليميراز المتسلسل المتداخل (Nested PCR) لدى مرضى التلاسيميا العراقيين المصابين بـ المقوسة الكوندية. جمعت عينة المرضى من مستشفى الكرامة التعليمي في بغداد، العراق، و80 حالة من الأصحاء، تتراوح أعمارهم بين سنتين و45 سنة، بمتوسط (15.387 ± 0.627) خلال الفترة من مارس إلى يونيو 2022 في العراق. استُخدمت الأجسام المضادة IgM/IgG للمقوسة في الكشف عن داء المقوسات باستخدام مقايصة الجسيمات الدقيقة الكيميائية الضوئية (CMIA). أظهرت النتائج وجود فرق معنوي ($P \leq 0.001$) في مستوى الأجسام المضادة IgG لمرض المقوسة الكوندية بين مجموعة السيطرة السلبية (0.5616±0.246 وحدة دولية/مل) ومجموعة السيطرة الإيجابية المصابة بداء المقوسات (24.985±6.101 وحدة دولية/مل)، كما كان الفرق معنويًا ($P < 0.001$) بين مجموعة السيطرة الإيجابية المصابة بداء المقوسات (35.59±8.336 وحدة دولية/مل) ومجموعة مرضى بيتا ثلاسيميا الكبرى المصابة بداء المقوسات (0.489±0.084 وحدة دولية/مل)، إلا أن جميع المجموعات كانت سلبية مصلية لأجسام مضادة IgM لمرض المقوسة الكوندية. تم الكشف الجزيئي باستخدام طريقة تفاعل البوليميراز المتسلسل المتداخل

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(Nested PCR) للكشف عن جين SAG2 في العينات المدروسة. أظهرت النتائج أن 25/6 (24.0%) من مرضى بيتا ثلاسيميا الكبرى المصابين بداء المقوسات أظهروا استجابة إيجابية لجين SAG2 باستخدام اختبار تفاعل البوليميراز المتسلسل المتداخل، بينما أظهرت مجموعات أخرى نتائج سلبية لجين SAG2 في نفس الاختبار. وختامًا، فإن مرضى بيتا ثلاسيميا العراقيين المشاركين في الدراسة الحالية مصابون بداء المقوسات المزمن. علاوة على ذلك، أظهرت النتائج وجود جين SAG2 للمقوسة الغوندية في عينات دم مرضى بيتا ثلاسيميا المصابين بداء المقوسات، بالإضافة إلى مجموعة الضبط الإيجابية.

الكلمات المفتاحية: المقوسة الكوندية، الثلاسيميا، جين SAG2، تفاعل البوليميراز المتسلسل

Introduction

Toxoplasma gondii is a parasite has single-celled that cause toxoplasmosis infection. It is one of the most common parasite diseases, affecting practically all warm-blooded species, including humans and pets. During pregnancy, this parasite can be vertically transmitted to the fetus, causing a variety of clinical problems in the baby. It is an opportunistic pathogen recurrence of the latent infection can cause death in congenitally infected fetuses, neonates, and immunocompromised patients [1-4]. *T. gondii* exists in three morphological forms: tachyzoites, bradyzoites, and sporozoites. It induces humoral immunity (H.I.) and cell-mediated immunity (C.M.I) responses, which are characterized as one of the most typical immunological kinds of this parasite infestation [5]. The parasite's intestinal phase life cycle occurs in the small intestine of cats, whereas the extra intestinal phase affects all intermediate hosts [6]. A group of hereditary disorders known as thalassemia causes a decreased rate of -or chain synthesis, which partially or completely suppresses the rate of hemoglobin synthesis [7].

Thalassemia is classified in two varieties: alpha and beta. Beta thalassemia major is an inherited disorder that may affect general health, gene mutations that result in low level and/or malfunctioning globin protein respectively, are the root causes of these disorders, one of these proteins might occasionally not exist at all carriers

of alpha or beta thalassemia trait exhibit minor symptoms depending on how severe the disease is, the human beta globin (HBB) gene, this is situated on chromosome 11 affects the structure and functionality of hemoglobin, alpha thalassemia can lead to consequences including hemolytic anemia or lethal hydrops fetalis, Given the constant mobility of individuals to different regions of the world, particularly western countries, sickle cell-beta thalassemia (HbS/-thal) is a good example of a combination of two frequent genetic anemias [8-9].

Nested polymerase chain is a modification of PCR designed to increase the sensitivity and specificity of the assay reaction. It involves the use of two primer sets directed against the same target and two successive PCR reactions. The first set of primers is designed to anneal to sequences upstream from the second set of primers, whereas the second set of primers is situated internally or nested with respect to the first set of primers. The first set of primers also called outer primers amplify a large fragment of the gene which is used as a template in the second round of PCR that targets a smaller region of the amplicon using the second set of primers also known as inner primers or nested primers [10-11]. The current study aimed to molecular detection of SAG2 gene by nested PCR method in Iraqi thalassemic patients infected with *T. gondii*.

1. Subjects

The study included 165 individuals suffering from thalassemia and 80 healthy individuals have been negative control group who attended to the Al-Karma Teaching Hospital in Baghdad, Iraq, these samples were collected during the period from March to June 2022. Ages ranging from (2-45) years, with mean (15.387 ± 0.627) years, after doctor's examination with application a necessary blood tests to detect thalassemia, serum samples were examined and diagnosed for anti-Toxoplasma IgM and IgG antibodies by using CMIA. Five milliliters of venous blood were withdrawn via using a sterile syringe from each patient. Two ml of whole blood was collected in a labeled EDTA tube in order to the molecular investigation, three ml was transferred into a fully labeled gel tube to separate the serum by centrifuge at 3000 rpm for 5 minutes and stored at -20 C° until used in CMIA detection.

2 .T. gondii diagnosis

1 - Serological diagnosis

CMIA was performed for the detection of both anti -*Toxoplasma* IgG/IgM antibodies in sera according to the manufacturer's instruction (Architect Toxo IgM/G kit -Abbott GmbH, Germany).

2 -DNA Extraction

DNA extraction followed the protocol proposed by Rossetti *et al.* (25) with some modifications. An aliquot of 500µl of each sample collected was centrifuged at 13000rpm for 10 min. and washed 3X with Tris-EDTA (TE) 1X. The pellet was resuspended in 100µl of TE 1X and thermoblock heated at 100°C for 10 min. After centrifugation, the supernatant was transferred to a new eppendorf tube and was added 5µl of resin (Sehgals Band

Prep Kit, Amersham Pharmacia Biotech) and 6M of sodium iodide 2:1 to it, then the eppendorf was stirred manually for 5 minutes and incubated at room temperature for 5 min. The tubes were centrifuged at 13000rpm for 1 min, the supernatant discarded, and 200µl of 70% ethanol was added, and then the tubes were centrifuged for 1 min. The sediment was dried at room temperature for 60 min, resuspended with 40 µl of TE 1X and incubated in a water bath at 50 °C for 10 min. After centrifugation for 1 min., the supernatant was transferred to a new tube and stored at -20 °C until the samples were processed. A negative control using TE was included during DNA extraction and it was manipulated as a biological sample to assess any type of contamination among the samples.

3-Amplification of genomic DNA of T. gondii

Nested PCR was performed on all DNA samples to amplify a fragment from SAG2 gene, which is present in 35 copies and is conserved in the T. gondii genome. The primer used in the first round of the PCR (inner primer pair) are F1 (55-CTGCTTGCGATTCTGTGTGT-3), and R1 (5-AGGTGCGGTCTTGAAAGCTA-3), respectively. The primer used in the second round (outer primer pair) are R2 (5-ACCTGGAGTCACTTCGGAGA-3). The PCR mixtures without DNA and with DNase-free water were used as negative controls to monitor for cross contaminations.

Statistical Analysis

The Statistical Analysis System- SAS (2022) program [12] was assessed Software Statistical Package for Science; statistical significance was determined by using L.S.D. test for quantitative dated. Results were expressed as mean $\pm$ S.D. Chi-square test was used to significant compare

between percentage ($P \leq 0.01$, $P \leq 0.05$) probability in this study.

Results and Discussion

Toxoplasmosis is a parasite protozoan disease that causes a variety of clinical disorders. It is usually asymptomatic in immunocompetent individuals, but it can cause substantial issues in

immunocompromised individuals and escalate to a life-threatening infection [13,14]. Furthermore, figure (1) shown that the group of thalassemic patients with toxoplasmosis has the highest level of anti-*Toxoplasma* IgG antibody ($60/165$) 24.985 ± 6.101 IU/ml followed by the group of non-thalassemic patients (positive control) ($25/80$) 35.59 ± 8.336 IU/mL. as shown in Figure (1).

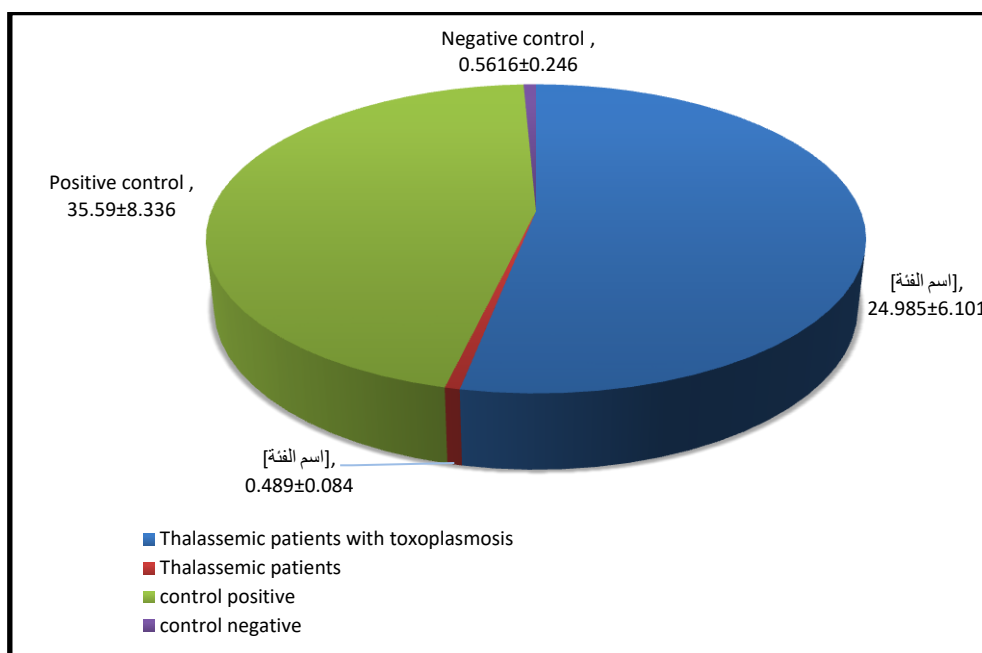


Figure (1): concentration of anti- Toxo IgG assay IU/mL in studied groups

The above results similar to the results of Yousef et al [15] found that 36/117(30.76%) of thalassemia patients and 41/205 (20%) of the healthy control was seropositive of anti-*Toxoplasma* IgG antibody in ELISA technique. Results of present study compatible with the results of El-Tantawy et al [16] that found prevalence of *Toxoplasma* IgG was 113/21(53.6%) in β - thalassemic children and 18% (18/100) in healthy control, furthermore, Hanifehpour et al [17] illustrated that 122/235 (51.9%) of β -thalassemia major patients and 82/235(34.8%) of healthy control were seropositive for *Toxoplasma* IgG antibody using ELISA assay,

also the current results of prevalence of *Toxoplasma* IgG were higher than the result of Abd El-Latif et al [18] that reported 10/100(10%) and 2/100(2%) of Egyptian thalassemia were seropositive for anti-*Toxoplasma* IgG and IgM antibodies respectively .

Results in table (1) showed that 6/25(24.0%) of β -thalassemic major patients infected with toxoplasmosis have positive response SAG2 gene by nested PCR assay, while others groups were negative for SAG2 in the same assay as shown in figure (2).

Table (1): Results of the nested PCR assay targeting SAG2 gene in studied groups

Groups	No. of samples in PCR	Positive	Negative	P-value
Thalassemic patients with toxoplasmosis	25	6 (24.00%)	19 (76.00%)	0.009 **
Thalassemic patients	20	0 (0.00%)	20 (100%)	-
Positive control	20	0 (0.00%)	20 (100%)	-
Negative control	20	0 (0.00%)	20 (100%)	-
P-value		0.0397 *	0.941 NS	---
** (P≤0.01).				

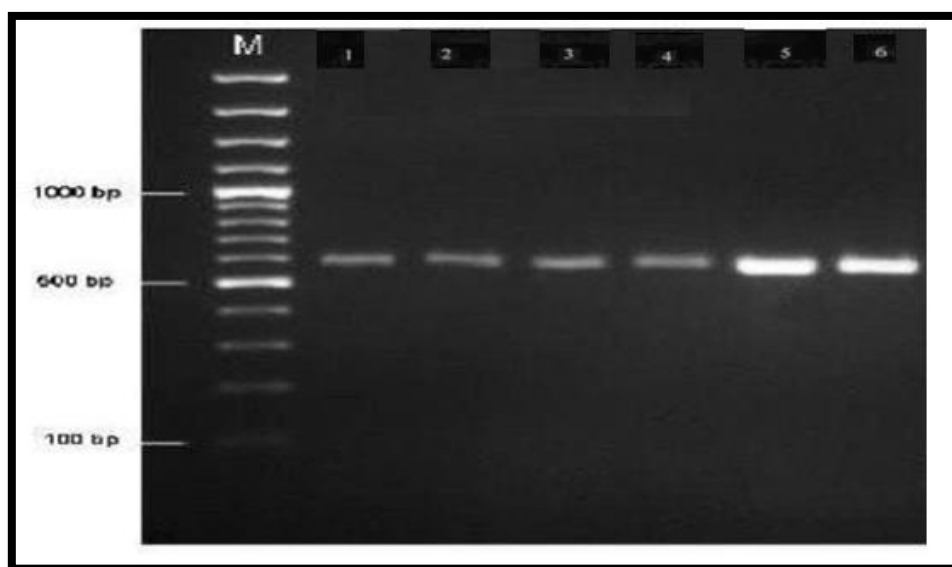


Figure (2): Electrophoresis of agarose (2%), 75 V for 2 hrs, Ethidium bromide at a concentration 2 µl, which shows the positive results of nested PCR technique for SAG2 gene (614 bp) of the group of of β-thalassemic major patients with toxoplasmosis M: Ladder DNA: 100 – 1000 bp

Because there are few studies that support the current results, the findings of Sabzevari et al [19] were similar to the current outcome which found that all 7 blood samples of Iranian patients infected with toxoplasmosis were negative for SAG2 detection in nested PCR technique, while these samples were detected positively for 529 bp RE in the same technique. This failure to amplify SAG2 gene were explained to poor sample preservation or too few parasites. Also, it was shown that the sensitivity of RE gene was more

than SAG2 gene for the diagnosis of the parasite in blood samples.

In contrast, Khanaliha et al [20] found that all 110 blood samples from Iranian patients with chronic toxoplasmosis (seropositive detection of IgG in ELISA) and 1/30 blood samples from Iranian healthy individuals tested positive for the SAG2 gene using PCR.

T. gondii cell surface is covered in surface antigens containing SAG2, which initially react with the target cell as part of its intracellular cycle.

It is the most abundant protein involved in adhesion and invasion. It has the ability to activate humoral immune responses and has been used to identify the acute phase and make a serological diagnosis of toxoplasmosis. SAG2 can also be displayed and processed by Major histocompatibility complex-I (MHC-I) to recognize CD8⁺ T cells. Furthermore, it allows for the rapid detection of various *T. gondii* genotypes from suspected clinical toxoplasmosis specimens [21].

Previous studies have shown that the *T. gondii* SAG2 gene is made up of three main clonal lineages, known as types I, II, and III, which are important in Europe and the United States, whereas the atypical genotype is common in South America and Asia. The nucleotide sequence analysis revealed that the SAG2 strain is unusual. It was also discovered that types II and III are the dominant genotypes [22].

Few results have been obtained in the molecular diagnosis of SAG2, the most important reason is that the infections are chronic in patients, this difference variation due to several factors differences in immune responses, The varying results from those regions may be due to differences in patients' number, type samples, demographics, history.

Conclusions

In conclusion, the Iraqi B-thalassemia patients of the current study were infected with chronic toxoplasmosis. Furthermore, result showed present of *T. gondii* SAG2 gene were detected in the blood samples of B-thalassemia patients with toxoplasmosis and positive control.

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