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Molecular detection of *Toxoplasma gondii* in a sample of Iraqi patients with acute leukemia and stem cell transplantation

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Abstract

BACKGROUND: Acute leukemia and allogenic bone marrow transplantation BMT are immunocompromised conditions which may be susceptible for many opportunistic infections or reactivation of latent infections like Toxoplasma gondii (*T.gondii*).

OBJECTIVES: The aims of study were to detect *T.gondii* in both acute leukemia patients and allogenic BMT recipients and to determine copy number of *T.gondii* in these groups in comparison to healthy individual as control group.

METHODS: Sixty one acute leukemia patients enrolled in a prospective study from 1st December 2016 to 1st June 2017. Forty eight of them evaluated while induction chemotherapy (group I), while the other 13 within 1 year post bone marrow transplantation-BMT-(group II). In addition to 30 apparently healthy individuals as (control group), blood samples were collected from all groups. *T.gondii* DNA was extracted and then measured by Taqman quantitative real-time PCR. Measurement IgG and IgM antibody specific to T. gondii was investigated also in the control group by an enzyme-linked immune assay (ELISA).

RESULTS: *T.gondii* parasitemia was detected in (8.3%) 4 out of 48 group I patient. While negative in group II and control group. The range of T.gondii load was (6.285×10^3-17.915×10^3) copy/ml, the mean of the copy numbers 11458.75± 5120.85.

CONCLUSION: *T.gondii* should be looked for Leukemic patients at least by routine serological test for early diagnosis and early treatment if indicated. Quantitative PCR is used to monitor post BMT patients at risk for *T.gondii* disease and for a timely start of preemptive therapy.

Keywords:

Acute leukemia, stem cell transplantation, *Toxoplasma gondii*

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Introduction

Leukemia is a malignant tumor of the hematopoietic system, which often has a poor prognosis.[1] It may be acute lymphoblastic leukemia (ALL) or acute myeloblastic leukemia (AML).[2,3] Chemotherapy and bone marrow transplantation (BMT) are the established therapeutic options for these patients, but there is an increased risk of infections,[4] secondary to disease and immunosuppressive therapy.[5,6]

Toxoplasma gondii is a protozoan parasite that causes a disease called toxoplasmosis. It is a very common parasitic infection in humans and other warm-blooded animals.[7] It is a wide-spread parasite

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reported to infect about one-third of the world population.[8] Toxoplasmosis in immunocompetent individuals is generally asymptomatic, but it often leads to serious pathological effects in immunocompromised patients.[9] It is also reported in immunocompromised patients with lymphatic leukemia,[10,11] and BMT,[12] where the tissue cysts may reactivate and cause disseminated infection like encephalitis and can be fatal for immunocompromised patients.[8,13] In Iraq, to the best of our knowledge, there is no such study on *T. gondii* in acute leukemia patients under chemotherapy or after postallogeneic BMT, and few studies investigated parasite infections in Iraq.

This study aimed to detect *T. gondii* in patients with acute leukemia after induction chemotherapy courses and post-BMT patients within the 1st year and to determine the copy number of *T. gondii* in comparison with apparently healthy individuals.

Methods

Study population

A prospective study was conducted from December 1, 2016 to June 1, 2017. Sixty-one patients were enrolled in this study, 48 (78.7%) of them had received an induction course of chemotherapy within 1 month of diagnosis as Group I. Those are 18/48 (37.50%) patients with acute lymphoid leukemia (ALL) and 30/48 (62.50%) with acute myeloid leukemia (AML). They collected from hematology ward at AL-Emamain AL-Kadhemain Medical City and Baghdad Teaching Hospital, Medical Complex, Iraq. The rest thirteen (21.3%) acute leukemia patients had assessed after allogeneic BMT within the 1st year of diagnosis as Group II and collected from the Bone Marrow Transplantation Center in the Medical Complex, Private Nursing House, Baghdad, Iraq. Another 30 apparently healthy individuals from volunteers and donors in the blood bank served as control group. Total number of ALL patients was 23 (37.7%) while 38 (62.3%) patients were with AML in both groups. A consent letter was obtained from all patients and controls were enrolled in the study. This study was approved by Institution Review Board of the College of Medicine-Al-Nahrain University. Clinical and laboratory data were obtained from all patients and controls, blood sample was collected from study groups, and 1 ml of whole blood samples was separated for parasitical DNA extraction. One milliliter of serum to measure immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody specific to *T. gondii* was investigated in the control group.

Parasitical DNA extraction

For parasitical DNA extraction from the whole blood samples, gSYNC™ Parasitical Nucleic Acid Extraction Kit (Geneaid, England) was used. One milliliter whole blood was used in parasitical DNA extraction, according to the manufacturer's protocol. For serum collection, the rest blood was centrifuged for the measure of the IgG and IgM specific antibody for *T. gondii* in the control group by enzyme-linked immunosorbent assay (ELISA).

Realtime polymerase chain reaction for measuring *T. gondii*

For the quantitative detection of *T. gondii*, ToxGon Dtec-qPCR Test F-100 Quantification Kit (Genetic PCR Solutions TM, Spain) was used. The real-time data are collected at the second step of the amplification cycle as demonstrated in Table 1. According to the manufacturer's instructions, *T. gondii* DNA copies were calculated according to the following formula:[14]

$$\text{Copy/ml} = \frac{\text{SC} \times \text{EV}}{\text{IV}}$$

SC = Sample Concentration (copy/μL)

EV = Elution Volume (μl)

IV = Isolation Volume (ml).

Serological test

Serum samples of control group were analyzed for anti-*T. gondii* IgG and IgM antibodies by a commercially available enzyme immunoassay toxoplasma IgG and IgM Kit (Toxo IgM and IgG μ CAPTURE Human Gesellschaft Germany) according to the manufacturer's instructions.

Statistical analysis

Microsoft Excel 2016 and Statistical Package for Social Sciences version 23 was used as software do the statistics. Most of the data were numerical so presented as mean ± standard deviation and comparison between means of study groups was done using unpaired Student's *t*-test. While the rest were nominal data which were presented as frequency and percentage, Fisher's exact test, Chi-square test, and Mann–Whitney test were used for comparison between frequencies of study groups. $P < 0.05$ was considered as statistically significant.

Results

The mean age was 37.27 ± 15.66 (range of 14–70 years), 29.77 ± 14.45 (range of 12–56 years), and 30.87 ± 10.58 (range of 14–53 years) for Group I, Group II, and controls, respectively; statistically, there was no significant difference ($P = 0.076$) between the mean of the two groups and control indicating that they were of a comparable age. The ratio of males was the predominant;

Table 1: *T. gondii* real time PCR amplification profile

Step	Time	Temperature
Activation[1]	15 min	950°
40 cycles		
Denaturation	15 sec	950°
Hybridization/Extension and data collection[2]	60 sec	600°

Table 2: Comparison of copy number in different study groups by ANOVA

Parameter	Group I copy/ml	Group II copy/ml	Control copy/ml
T. gondii copy no.	12.985×10^3	Negative	Negative
	17.915×10^3		
	8.650×10^3		
	6.285×10^3		
Mean	11458.75	Negative	Negative
SD	5120.85		
Range	6.285×10^3-17.915×10^3		

56.3% (27/48), 76.9% (10/13), and 56.7% (17/30) in both groups and control, respectively.

Quantitative real-time polymerase chain reaction (QRT-PCR) run demonstrated positive parasitemia in 4 out of 48 (8.3%) in Group I, but neither of the Group II patients nor any of the control group was positive.

The range of *T. gondii* load was $6.285 \times 10^3 - 17.915 \times 10^3$ copy/ml in Group I while negative in Group II and control group. The mean of the copy numbers in Group I was 11458.75 ± 5120.85 as shown in Table 2.

During collection of samples, 16 samples were obtained pre- and post-chemotherapy, two of these samples was negative before chemotherapy (induction) and positive after chemotherapy in QRT-PCR and was confirmed by ELISA as shown in Table 3.

The results of control group for anti-*Toxoplasma gondii* immunoglobulin M and immunoglobulin G by enzyme-linked immunosorbent assay

All control groups in the study had negative result in QRT-PCR and anti-*T. gondii* IgM. Regarding the control group, 5 (16.67%) samples out of the 30 serum samples were positive for anti-*T. gondii* IgG as shown in Table 4.

There was no significant relation between *T. gondii* parasitemia and age group in acute leukemia ($P = 0.187$). It is demonstrated that there was no significant relationship between parasitemia and sex ($P = 0.594$). *T. gondii* parasitemia was detected in 2 males and 2 females. Furthermore, there was no significant correlation between positive *T. gondii* parasitemia

Table 3: Toxoplasma methods of detection in relation to phases of chemotherapy

Patients (n=2)	Pre-chemotherapy (induction)	Post-chemotherapy (induction)
T. gondii by PCR copy/ml	Negative	Positive
	Negative	Positive
IgG *T. gondii* by ELISA UI/mL	3.1 UI/MI	21.1 UI/MI
	5.7 UI/MI	26 UI/MI

Table 4: The results of control group for anti-*Toxoplasma gondii* IgM and IgG by ELISA

Control group	Negative No. (%)	Positive No. (%)	Total No. (%)
T. gondii by PCR	30 (100)	0 (0)	30 (0)
IgM *T. gondii* by ELISA UI/MI	30 (100)	0 (0)	30 (100)
IgG *T. gondii* by ELISA UI/MI	25 (83.33)	5 (16.67)	30 (0)
Result UI/mL	0.9 0.7	1.21 1.6	1.02

with the residence of the patient ($P = 337$). However, the occupation showed a significant association with *T. gondii* parasitemia ($P = 0.01$) as in Table 5.

Comorbidity and history of blood transfusion in relation to *Toxoplasma gondii* reactivation

Both comorbidity and history of blood transfusion showed no statistical significant association with toxoplasmosis parasitemia ($P = 1.000$, 0.575, and 0.423, respectively) as in Table 6. Similarly coinfection with hepatitis B virus showed no statistical relationship with toxoplasmosis parasitemia ($P = 0.76$).

Relationship between the *Toxoplasma gondii* parasitemia with the hematological parameters

Table 7 shows the comparison of values of hematological parameters between the *T. gondii* positive and negative patients (Group I and Group II);there was significant association between *T. gondii* parasitemia with leukopenia and neutropenia, ($P = 0.017$ and $P = 0.037$), respectively. While there was no significant relation between lymphocytes count and hemoglobin with positive toxoplasmosis, there was significant association between platelet count with positivity toxoplasmosis ($P = 0.013$).

Discussion

In this study, *T. gondii* was investigated in blood samples of patients with acute leukemia (Group I), PBMT (Group II), and control using QRT-PCR; most patients and all photobiomodulation therapy (PBMT) and control had negative *T. gondii*. Only 4 out 48 (8.33%) with acute leukemia patients were positive for *T. gondii* by QRT-PCR; this result is within the range in comparison to other studies included in the meta-analysis, ranging from 6.8% to 21.6% by Huang *et al.* 2016.[15] One of the most critical problems in leukemia is infectious diseases which

Table 5: Comparison of the demographic data in relation to *T. gondii* positivity

Parameter		Group I *n*=4 no. (%)	*P*
Age (yr)	<20	2 (50)	0.187
	20-39	2 (50)	
	40-59	0 (0)	
	≥ 60	0 (0)	
Sex	Male	2 (50)	0.594
	Female	2 (50)	
Residence	Baghdad	1 (25)	0.337
	Other	3 (27)	
Occupation	Student	2 (50)	0.01
	Employee	0 (0)	
	Free work	0 (0)	
	housekeeper	2 (50)	

Table 6: Frequency of co-morbidity and history of blood transfusion in relation to *T. gondii* positivity

Parameter		Group I *n*=4 No. (%)	*P*
DM	Present	0 (0)	1.000
	Absent	4 (100.0)	
HT	Present	0 (0)	0.575
	Absent	4 (100.0)	
Blood transfusion	Yes	4 (100.0)	0.423
	No	0 (0)	
HBV	Present	0 (0)	0.761
	Absent	4 (100.0)	

Table 7: Relationship between *T. gondii* positivity with haematological parameters

Parameter		*T. gondii* Negative *n*=57	*T. gondii* Positive *n*=4	*P*
WBC (*10^3/µl)	Mean	9.13	1.33	0.017
	SD	17.58	0.92	
	Median	4.10	1.41	
	Range	0.26-86.43	0.2-2.3	
Neutrophils (*10^3/µl)	Mean	3.69	0.58	0.037
	SD	6.08	0.65	
	Median	1.50	0.28	
	Range	0.01-30.57	0.2-1.55	
Lymphocytes (*10^3/µl)	Mean	3.23	0.70	0.094
	SD	7.42	0.52	
	Median	1.30	0.80	
	Range	0.23-40.39	0.01-1.2	
Hemoglobin (g/dl)	Mean	9.38	8.85	0.884
	SD	3.00	1.63	
	Median	8.80	8.60	
	Range	4.2-16.4	7.2-11.0	
Platelets (*10^3/µl)	Mean	116.84	22.50	0.013
	SD	94.05	4.65	
	Median	92.00	23.50	
	Range	7-355	16-27	

may lead the patient succumbs to sudden death. The active infection may alter the normal immune response of the host. Granulocytes and macrophages play a main role in immune surveillance in innate immune system.[16] This result may suggest that leukemic patients under immunosuppressive condition had been infected with *T. gondii* before initiation of leukemia development. Therefore, the immunosuppressive therapy may provide the reactivation of toxoplasma disease.[17] According to the result of this study, two of these samples were negative before chemotherapy (induction) and positive after chemotherapy in QRT-PCR and were positive pre- and post-chemotherapy by ELISA.

All the controls in this study were negative for *T. gondii* by QRT-PCR. Moreover, these results in apparently healthy controls were in agreement with meta-analysis by Huang *et al.* 2016[15] as shown in Table 8. The total population infected with *T. gondii* was 5/30, 16.66% in the control group of this study by ELISA. This result is similar to the study in the central Mexican State of Jalisco by De *et al*., 2005, where researcher found that 17.8% of high-school students were positive for T. gondi,[18] and the prevalence of toxoplasmosis in healthy blood donors examined was 18.7%, in Iraq, Kirkuk city by ELISA test by Mohammad and Jasim, 2017.[19] This result of the control group is much lower than that reported in Baghdad; the seropositive toxoplasmosis by ELISA was 30.25% by Mahmood *et al.* 2013[20] while it was 27.8% for IgG ELISA in Iraq, Thi-Qar by Hadi *et al*., 2010.[21] The explanation of previous percentage differences stated by different researchers may be related to the different geographical area within one country and within the same city.[8] These differences may be related to several other factors, including difference sample size as well as cultural level, nutritional habits, age, and rural or urban area.[22]

The results of this study showed that all patients with PBMT were negative for *T. gondii* by QRTPCR, which differs from other studies; however, the percentage of toxoplasmosis rate are very low in those studies such as Martino *et al.*, 2000[23] which showed that the T. *gondii* infection was 8 out of 1,000 patients and Busemann *et al*., 2012[24] which showed that the toxoplasmosis after allogeneic stem cell transplantation was 3/155, 1.9%. This difference may be due to the small number of cases in the present study. Toxoplasmosis represents a rare but potentially life-threatening complication in allogeneic hematopoietic stem cell transplantation.[25]

Toxoplasma gondii infection and demographic data

In the current study, it appeared that there was no significant correlation between age and toxoplasmosis. In the age range <20 years, two male patients were positive for *T. gondii* similar to blood donors with <20 years of age in Mexican obtained by De *et al.*, 2005;[18] in contrast, in this study, the highest positive rate was seen in the age of 21–30 years by Mahmood *et al*., 2014,[26] and the prevalence rate increases with age as described by

Table 8: Characteristics of studies included in the meta-analysis of *T.gondii* and current study

First author	Year	No. and type of cases	Cases		No. and type of controls	Controls		Method for diagnosis	Target of detection	Area
			Positive	Negative		Positive	Negative			
Yang	2005	46 acute leukemia	8	38	20 healthy controls (PB)	0	20	PCR	*T.gondii* DNA	China
Chang	2007	58 acute leukemia	4	54	20 healthy control (PB)	0	20	PCR	*T.gondii* DNA	China
Current study	2017	48 acute leukemia	4	44	30 healthy control (PB)	0	30	PCR	*T.gondii* DNA	Iraq

*PB – Population-based; **PCR – Polymerase chain reaction

Montoya and Liesenfeld, 2004,[13] RobertGangneux and Dardé, 2012,[27] and Shimelis *et al*., 2009.[28] Hypothesis is that the rate of toxoplasma infection increases with age. This may be because of the increased risk of exposure to infection source with age,[29] and the other two cases were in the age group 20–39 years, positive for *T. gondii*. This result was in agreement with the other studies, Rezanezhad *et al*., 2017[30] and Mohammad and Jasim, 2017.[19] However, Alvarado-Esquivel *et al*., 2007[31] reported that this age group that range between 30 and 39 years was more commonly infected with toxoplasmosis.

Regarding gender, no statistical difference was found; the four toxoplasmosis patients were two males and two females; these results demonstrate that males and females have the same probability of contracting *T. gondii* infection, which has been demonstrated by other studies from Korea by Yang *et al*., 2000,[32] Hatam *et al*., 2005,[33] Brazil by Lopes *et al*., 2005,[34] and in the systematic review and meta-analysis of *T. gondii* infection seroprevalence in Iran by Daryani *et al*., 2014.[35] There was no significant difference in the seroprevalence rate between male and female patients.

Occupation and toxoplasma infection showed significant correlations in this study (P = 0.01); these findings were in agreement with the results obtained by Salahi-Moghaddam and Hafizi, 2009.[36] Two housekeepers were toxoplasma positive. This may be interpreted by continuous exposure of women to the risk factors of *T. gondii* infection through their routine house works such as minced contaminated meat products, gardening that cause direct contact with cats' feces-contaminated soil especially in rural area, eating of unwashed vegetables and fruits, and drinking of municipal water from contaminated reservoirs; this may explain these significant correlations.[37] The other two positive toxoplasma patients were students; this may be due to many reasons such as eating outside home or dealing with many sources of infection because students in the study period are active and deal with their surroundings and this may expose them to infection.

Toxoplasma gondii infection and comorbidity

There was no statistical significance of comorbidity with both diabetes and hypertension in this study which might be because of the small sample size in this study; however, the prevalence rates of diabetes and toxoplasmosis are higher in Iran studies done by Shirbazou *et al*., 2013[38] and Siyadatpanah *et al*., 2013,[39] and many countries around the world according to the studies done by Gokce *et al*. 2008,[40] Barbosa *et al*. 2009,[41] and Sarkar *et al*. 2012.[42]

Toxoplasma gondii and infection hematological parameters

There was statistically significant association between leukopenia and *T. gondii* parasitemia (P = 0.017). Chemotherapy that is used to treat acute leukemia induced leukopenia.[43] The significance of this association can be assumed that immune-compromised status of patients due to disease or chemotherapy will result in subsequent host defenses impairments.[44-47] Thus, it provide an opportunity for the inactive latent *T. gondii* to get re-activation.[18]

There was significant relation between neutrophils count and *T. gondii* reactivation (P = 0.037). This is possibly explained by the fact that normal levels of neutrophils are essential to prevent infections.[48] Therefore, new infection or reactivation of *T. gondii* is more in patients with acute leukemia because normal hematopoiesis is replaced by abnormal maturation and deregulated proliferation of leukocytes.[3] Coupled with significant bone marrow infiltration, this leads to decreased production of normal granulocytes resulting in neutropenia and impaired granulocyte function. In addition, the presence of a large number of immature myeloid cells can inhibit antigen-specific T cell response.[49] Treatment with standard induction regimens results in prolonged neutropenia that can last weeks, rendering the host highly susceptible to infections.[3] Furthermore, polymorphonuclear leukocyte function may be adversely affected by several chemotherapeutic medications such as high-dose glucocorticoids and vincristine.[50] The risk of severe infections is related to the degree and duration of neutropenia.[3]

There was significant association between platelet count and positivity toxoplasmosis (P = 0.013). Thrombocytopenia arises from ineffective production of platelets by bone marrow, example is the processes occupying bone marrow (e.g., leukemia, lymphoma, and multiple myeloma) and drug-induced thrombocytopenia caused by direct myelosuppressive effect (e.g.,

chemotherapy-induced thrombocytopenia),[51] and severe thrombocytopenia may be associated with toxoplasmosis.[51] Therefore, this may be the cause of relation between parasitemia and thrombocytopenia because patients with acute leukemia were treated with chemotherapy and toxoplasmosis infection.

Conclusion

T. gondii should be looked for leukemic patients at least by routine serological test for early diagnosis and early treatment if indicated. Quantitative PCR is used to monitor post-BMT patients at risk for *T. gondii* disease and for a timely start of preemptive therapy.

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Conflicts of interest
There are no conflicts of interest.

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