Toxoplasmosis in adult patients with haematologic malignancy: Seroprevalence of anti-*Toxoplasma* antibodies and molecular diagnosis

Original Article

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ABSTRACT

Background: Serological diagnosis of toxoplasmosis in patients with haematological malignancy (HM) is challenged by the impaired antibody response; meanwhile, molecular testing is necessary to demonstrate reactivation.
Objective: Study of detection rates and risk factors for toxoplasmosis in HM patients using serological and molecular methods.
Subjects and Methods: The study included 40 adult patients with HM, and 40 age and sex-matched healthy individuals. Data on risk factors of toxoplasmosis were collected. Serologic testing for anti-*Toxoplasma* IgG and IgM antibodies was performed using ELISA. Blood samples were examined molecularly for detection of *T. gondii 529re* gene.
Results: Seropositive rate was significantly (P=0.003) higher among patients (75%) compared to healthy individuals (42.5%). However, seropositive patients displayed lower anti-*Toxoplasma* IgG concentrations compared to healthy individuals (P<0.05). Positive PCR results were obtained in 13 patients (32.5%) and one healthy individual (2.5%) with a significant difference (P=0.0004). Seropositive patients showed higher PCR positive rate (36.7%) compared to seropositive healthy individuals (5.9%) (P<0.05). No statistically significant association was recorded between toxoplasmosis and the collected data for risk factors.
Conclusion: Patients with HM are at high risk of toxoplasmosis and/or reactivation of latent infection. The combination of serology and PCR is more accurate to reach a definite diagnosis. Follow-up is necessary to prevent the development of life-threatening toxoplasmosis in these patients.

Keywords: Egypt; haematologic malignancy; immunosuppression; molecular diagnosis; seroprevalence; toxoplasmosis.

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INTRODUCTION

Patients with HM, including those with acute myelogenous leukemia, and those who have undergone hematopoietic stem cell transplantation or are treated with aggressive immunosuppressive regimens are at high risk of opportunistic infections. Prompt correct diagnosis is essential for effective management of possible infections[1,2]. On the other hand, *T. gondii* is a widespread obligate intracellular protozoan parasite causing opportunistic infection in immunocompromised patients. Human infection is caused by ingestion of cysts in undercooked meat or oocysts in contaminated food or water. Infection may be also transmitted congenitally or through organ transplantation[3]. After initial dissemination, the parasite can evade the immune system and persist throughout the host life within dormant cysts, predominantly in the brain, retina, and muscles[4].

Infection is generally asymptomatic and has little clinical relevance in immunocompetent subjects[5]. Reactivation of latent cysts may occur in immunocompromised patients when the immune system fails to maintain an efficient T helper-1 immune response. This can lead to cerebral or disseminated disease[6]. The incidence of reactivated toxoplasmosis is closely related to *T. gondii* seroprevalence in the population. The duration and degree of immune suppression are strong predictors of reactivation[7]. Primary toxoplasmosis in immunocompromised patients is also life-threatening due to the involvement of multiple organs[8].

Definitive diagnosis of toxoplasmosis is made by isolation of the parasite from blood or body fluids, and by histological examination of affected tissues using specific stains[7]. Since these methods are difficult and time-consuming, the classical diagnosis of

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toxoplasmosis usually relies on the detection of specific antibody responses[9]. However, serological methods also have poor efficiency in immunocompromised patients due to delayed or impaired antibody production[3,10]. Molecular diagnosis to detect *T. gondii* DNA using primers specific to different regions of the parasite genome was described[11,12]. The ability of molecular methods to detect low parasite burden is valuable, as *Toxoplasma* can circulate inconstantly and at low concentrations with potential reactivation[13]. Thus, the use of PCR assays for detection of *T. gondii* DNA in blood or body fluids is strongly recommended as an additional diagnostic method especially in immunocompromised patients[12,13]. The PCR protocols targeting the highly conserved *T. gondii* repetitive DNA sequences *529re*, *b1* gene, and internal transcribed spacer-1 (ITS-1) were widely used[11-13]. Molecular PCR assays that amplify *529re* gene are extremely sensitive and specific for molecular detection of *T. gondii*[12,14].

The present study aimed to evaluate detection rates of *T. gondii* infection in patients with HM compared to healthy individuals using seroprevalence of anti-*Toxoplasma* antibodies and molecular diagnosis, and to shed light on possible risk factors of toxoplasmosis in immunocompromised patients with HM.

SUBJECTS AND METHODS

This case-control study was conducted in Medical Parasitology Department, Medical Research Institute, University of Alexandria during the period from February 2019 to October 2019.

Study design: The study included patients with HM and healthy controls. Seroprevalence of anti-*Toxoplasma* antibodies and molecular diagnosis were used. Risk factors for toxoplasmosis and its relation to type of malignancy and chemotherapy were assessed.

Sample size calculation: This was calculated using Epi info 7 (Centers for Disease Control and Prevention; Atlanta, GA, USA). Based on *Toxoplasma* prevalence rate of 65% in the Egyptian population[15] and with a risk ratio of 1.5 in cancer patients[16,17], the sample size (40 in each group) was considered adequate at 95% confidence interval with a statistical power of 90%.

Study population: All patients were recruited from the Haematology Department, Medical Research Institute (Alexandria University, Egypt). Healthy individuals were recruited from those accompanying the patients and from workers in the Medical Research Institute.

Data collection: All participants were interviewed using a preformed structured questionnaire to collect demographic characters. Other data included the lifestyle habits that pertain to exposure to toxoplasmosis such as contact with cats and consumption of undercooked meat. The medical history of patients including the type of malignancy, blood transfusion, and the treatment received was also recorded.

Sample collection: A single venous blood sample (~5 ml) was drawn from each participant and divided into two tubes; a plain tube and a tube with an anticoagulant. Samples were immediately transported to the laboratory of the Parasitology Department, Medical Research Institute, Alexandria University. The first part of the blood sample was centrifuged at 3000 rpm for 5 min to obtain sera. Clear, non-hemolyzed sera were divided into two clean labeled Eppendorf tubes and stored at -20°C till used for seropositivity testing. The second part of the blood sample was stored at -80°C till used for DNA extraction and molecular testing.

Serological diagnosis[15,17]: The separated sera were tested for detection of anti-*Toxoplasma* IgM and quantitative determination of anti-*Toxoplasma* IgG concentration using commercially available ELISA assays (Biocheck Inc., Foster City, California, 94404 USA). The tests were performed according to the manufacturer's instructions. For IgG results, samples with concentration ≥32 international units (IU)/ml were considered positive.

Molecular diagnosis[18]: DNA was extracted from the anticoagulated blood samples using QIAamp DNeasy Mini kit (Qiagen cat. no. U.S.69504 and 69506) according to the manufacturer's directions. Amplification of *Toxoplasma 529re* gene was performed in a thermal cycler (Beco, Germany) using the primers TOXO 4: 5'CGCTGCAGGGAGGAAGACGAAAGTTG3' and TOXO 5: 5′CGCTGCAGACACAGTGCATCTGGATT-3′[18]. The primers were synthesized by Fermentas (Fermentas UAB, Lithuania). The PCR reaction was performed using Red Taq master mix (Bioline, UK). The amplification steps included initial denaturation step at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 30 sec, and extension at 72°C for 45 sec, and a final extension step at 72°C for 10 min. The PCR final products were separated in 1% agarose gel electrophoresis at 100 v for 30 min. The bands of the expected size were visualized against a 100 bp DNA ladder on a UV transilluminator. Each PCR round included a negative control of nuclease-free water and a positive control of DNA extracted from *T. gondii* RH strain maintained in the Parasitology Department, Medical Research Institute, Alexandria University.

Statistical analysis: The obtained data were analyzed using the Statistical Package for the Social Sciences (SPSS) software version 20 (Chicago, IL, USA). Categorical variables were compared using Pearson's Chi-squared test. The t-test was used to analyze the difference between the means of quantitative data of two groups. The agreement between serology and PCR was studied by Cohen's Kappa agreement test. A *P* value of less than 0.05 was considered statistically significant.

Ethical consideration: The study protocol was approved by the Ethical Committee of the Medical Research Institute, University of Alexandria. All patients and healthy subjects were asked to voluntarily participate and were offered information about the procedure and objectives of the study. Informed consent was obtained from each participant before inclusion in the study. Patients and healthy individuals with positive serological or molecular test results were advised to consult physicians for treatment.

RESULTS

Characteristics of the study population: The mean age of patients was 40.0±15.88 years (range: 17-76 years) and the mean age of the healthy individuals was 38.6±15.69 years (range: 18 to 76). In both groups, 65% of participants were aged ≤40 years. Males constituted 47.5% of patients and 40% of healthy individuals. Among the 40 HM patients, 19 (47.5%) had acute myeloid leukemia (AML) and 12 (30%) had acute lymphoid leukemia (ALL). Less common forms of HM among participating patients were chronic lymphoid leukemia (CLL) (5 patients), chronic myeloid leukemia (CML) (3 patients), and hairy cell leukemia (one patient). Collectively, 75% of the patients were on cytotoxic chemotherapy and 25% had not yet received treatment. The main symptoms among HM patients were fever, unexplained fatigue, weight loss, and anemia-related symptoms. None of the patients had neurological or pulmonary symptoms.

Anti-*Toxoplasma* antibodies in HM patients and healthy individuals: Serological testing revealed that 27 HM patients (67.5%) and 16 healthy participants (40%) had anti-*Toxoplasma* IgG (P=0.013) with no detectable IgM. Anti-*Toxoplasma* IgM was detected together with IgG in 3 HM patients (7.5%) and one healthy participant (2.5%) with no significant difference. The overall *T. gondii* seropositive rate was significantly higher among HM patients compared to the healthy participants (75% versus 42.5%, P=0.003). Molecular diagnosis gave positive results in 13 out of the 40 HM patients (32.5%) and one of the 40 healthy participants (2.5%) with a highly statistically significant difference (P =0.0004) (Table 1).

Among patients with different types of HM, *T. gondii* seropositive rate ranged from 58.3% to 100% and the PCR positive rate ranged from 21.1% to 100%. Neither serology nor PCR positive results showed a significant association with the type of HM (Table 2).

Seropositive HM patients displayed significantly lower mean±SD anti-*Toxoplasma* IgG concentrations (290.69±147.8 IU/ml) compared to seropositive healthy individuals (550.6± 281.95) (P=0.0001). Seropositive patients were more likely to be PCR positive compared to seropositive healthy individuals (36.7% versus 5.9%, P<0.05) (Table 3).

Serology versus PCR for detection of toxoplasmosis: Positive PCR results (Figure 1) were obtained in a relatively greater proportion of HM patients with

Table 1. Serological and molecular detection of *T. gondii* in patients with haematological malignancy compared to healthy individuals.

	Patients (n=40)	Healthy individuals (n=40)	Statistical analysis
	No. (%)	No. (%)	*P* value
Anti-*Toxoplasma* IgG only	27 (67.5)	16 (40.0)	0.013*
Anti-*Toxoplasma* IgG+ IgM	3 (7.5)	1 (2.5)	0.304
Total seropositivity	30 (75.0)	17 (42.5)	0.003*
PCR	13 (32.5)	1 (2.5)	0.0004*

P values calculated by Chi-square test; ***:** Statistically significant (P≤0.05).

Table 2. *T. gondii* among the examined patients according to the type of haematological malignancy.

Type of haematological malignancy	No. examined	Serology	*P* value	PCR	*P* value
		No. (%)		No. (%)	
Acute myeloid leukemia	19	15(78.9)		4(21.1)	
Acute lymphoid leukemia	12	7 (58.3)		6 (50.0)	
Chronic lymphoid leukemia	5	5 (100)	0.4	1 (20)	0.263
Chronic myeloid leukemia	3	2 (66.7)		1 (33.3)	
Hairy cell leukemia	1	1 (100)		1 (100)	

P values calculated by Chi-square test.

Table 3. Anti-*Toxoplasma* IgG concentration and PCR results among seropositive haematological malignancy patients and healthy individuals.

Test	Seropositive patients (n=30)	Seropositive healthy individuals (n=17)	*P* value
Mean±SD of IgG concentration (IU/ml)	290.69 ± 147.81	550.62 ± 281.95	**0.0001*@**
PCR positive No. (%)	11 (36.7)	1 (5.9)	**0.007*#**

***:** Significant (P≤0.05), **@:** *P* value calculated by *t*-test, **#:** *P* value calculated by Chi-square test.

detectable IgG and IgM antibodies (2 out of 3 patients, 66.7%) compared to those who had IgG with no detectable IgM antibodies (9 out of 27 patients, 33.3%). Twenty% of seronegative patients (2 out of 10 patients) were PCR positive. There was no significant association between PCR results and detection of anti-*Toxoplasma* IgG or IgM (Table 4). Serology (IgG with or without IgM) showed a slight agreement with PCR for detection of toxoplasmosis in HM patients (k= 0.011). Among the healthy individuals, the single PCR positive individual was IgG positive-IgM negative (data not shown).

Factors associated with *T. gondii* infection among the examined patients: Using ELISA as the diagnostic method, all variables showed insignificant results. It was observed that *T. gondii* seropositivity rates were slightly higher among those above 40 years (78.6%) compared to younger patients (73.7%). Females also had a slightly higher rate than males (76.2% versus 73.7%). Contact with cats was related to a higher rate of infection (81.8% versus 66.7%). Consumption of undercooked meat did not affect seropositivity rates. Regarding clinical history, it was found that the chronic form of malignancy, receiving chemotherapy, and blood transfusion were related to higher seropositivity rates. However, none of these parameters were significantly associated with *T. gondii* infection (Table 5).

Using PCR, similar age and gender differences were observed (P>0.05). Contact with cats was associated with a higher rate of positive PCR results, although this did not reach the level of statistical significance (*P* = 0.053). Patients who did not receive chemotherapy were more likely to be PCR positive (50%) compared to those receiving chemotherapy (26.7%), but this was not statistically significant. The type of HM, whether acute or chronic, and the history of blood transfusion were not associated with significant elevation in *T. gondii* infection rate (*P* = 0.564).

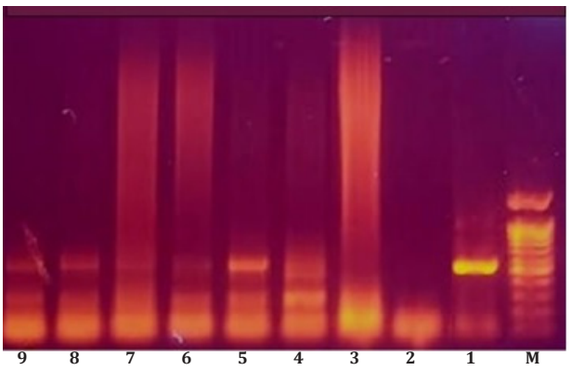

Fig. 1. PCR amplification of *T. gondii 529re* gene (529bp) using TOXO 4 & TOXO 5 primers in blood samples. **Lane M:** DNA ladder, **lane 1:** positive control, **lane 2:** negative control, **lanes 3,4:** negative samples, **lanes 5-9:** positive samples.

Table 4. Association between IgG and IgM ELISA and PCR results for detection of toxoplasmosis among patients with haematological malignancy.

	PCR		*P* value
	Positive No. (%)	Negative No. (%)	
Positive IgG and negative IgM	9 (33.3)	18 (66.7)	0.3139
Positive IgG and positive IgM	2 (66.7)	1 (33.3)	
Negative IgG and negative IgM	2(20)	8 (80)	
Total	13 (100)	27 (100)	

P value calculated by Chi-square test.

Table 5. *T. gondii* infection diagnosed by serology and PCR among patients with haematological malignancy according to plausible risk factors.

Characteristic variable		No. examined	Serology positive No. (%)	*P* value	PCR positive No. (%)	*P* value
Age (Y)	**≤40**	26	19 (73.7)	0.702	9 (34.6)	0.697
	>40	14	11 (78.6)		4 (28.6)	
Gender	**Male**	19	14 (73.7)	0.855	6 (31.6)	0.906
	Female	21	16 (76.2)		7 (33.3)	
Contact with cats	**Yes**	22	18 (81.8)	0.271	10 (45.5)	0.053
	No	18	12 (66.7)		3 (16.7)	
Consumption of under cooked meat	**Yes**	32	24 (75)	1	11 (34.4)	0.613
	No	8	6 (75)		2 (25)	
Receiving chemotherapy	**Yes**	30	23 (76.7)	0.673	8 (26.7)	0.172
	No	10	7 (70)		5 (50)	
Blood transfusion	**Yes**	22	23 (76.7)	0.271	8 (34.6)	0.564
	No	18	7 (70)		5 (27.8)	
Malignancy condition	**Yes**	31	18 (81.8)	0.274	10 (32.3)	0.952
	No	9	12 (66.7)		3 (33.3)	

P values calculated by Chi-square test

DISCUSSION

The intracellular protozoon, *T. gondii* is recognized as an opportunistic parasite in immunocompromised patients[3,10]. Results of the present study revealed that *T. gondii* seropositive rate ranged from 58-100% in patients with different forms of HM. The overall seropositive rate was significantly higher in HM patients compared to healthy individuals. In Egypt, seroprevalence rates as high as 90% and as low as 20% were previously reported among patients with other forms of malignancy[19-21]. Similar studies conducted worldwide reported concordant results. In Iran, Gharavi *et al.*[22] detected *T. gondii* IgG antibodies in 56.4% of leukemia patients and 42.4% of the control group. A meta-analysis incorporating nineteen studies conducted in China with a total of 4493 patients and 6797 controls concluded that the overall *T. gondii* seroprevalence was significantly higher in the population with cancer compared to those without (20.59% vs 6.31%)[23]. The variations in *Toxoplasma* seroprevalence rates among different countries may be attributed to different social and cultural habits, distinct environmental and geographical factors, and different livestock rearing practices[7].

The higher seropositivity of anti-*T. gondii* antibodies among HM patients compared to healthy individuals may be attributed to the weakened host immunity. In acute leukemia, immune dysfunction results from abnormal maturation and dysregulated proliferation of leukocytes and significant bone marrow infiltration. Additionally, the presence of a large number of immature myeloid cells can inhibit antigen-specific T cell responses[24]. In chronic leukemias, the main underlying pathology is the accumulation and slow proliferation of mature appearing but functionally incompetent leukocytes. The B cell defects lead to hypogammaglobulinemia involving the three classes; IgG, IgA, and IgM[25,26]. Impaired functions of natural killer cells, neutrophils, monocytes, and macrophages were recorded[24]. The T cell defects interfere with the initiation and maintenance of the immune response[27]. In HCL that has a slow progressing course, neutropenia and monocytopenia increase susceptibility to infection[24], and all treatment regimens can further exacerbate immunosuppression in all types of HM[2].

A positive IgG test merely indicates that the host was previously infected whereas positive IgM results indicate a relatively recent infection. In the case of immunodeficiency, patients with latent toxoplasmosis are at risk for reactivation whereas recently infected patients are at risk for acute disseminated toxoplasmosis, in which diagnosis of infection is an emergency[8,28]. Although serologic detection of IgM and high IgG titers could predict an acute infection, and could, therefore, be valuable in emergency diagnosis, the application is limited in immunocompromised patients due to reduced antibody production[7,29]. This was confirmed in the present study by the significantly lower IgG concentration observed in seropositive patients with HM compared to controls. This reduced antibody response represents a challenge for serological diagnosis of toxoplasmosis. Therefore, molecular assays for DNA detection in clinical samples is essential[30].

In the present study, PCR analysis revealed detection of *Toxoplasma* DNA in 32.5% of patients compared to 2.5% of controls with a statistically significant difference; however, PCR positive rate was not significantly related to the type of malignancy. Generally, detection of *T. gondii* in clinical samples confirms the presence of parasites which can be due to primary, reactivated, or chronic infection[31]. Although the presence of *Toxoplasma* DNA in blood suggests parasitemia, its clinical significance in immunocompromised patients is still unclear. *T. gondii* DNA was detected by PCR in blood samples of 80% of patients with cerebral toxoplasmosis[32]. *Toxoplasma* DNA may be also detected in the blood of immunocompromised patients who had no localizing signs and symptoms[33,34]. The detection of *T. gondii* may precede the onset of disease reactivation, which indicates that PCR monitoring of peripheral blood may contribute to early diagnosis[35].

Several reports suggested that PCR amplifying the *529re* gene is extremely sensitive and specific for molecular detection of *T. gondii*[31]. Most seropositive controls (16 out of 17 subjects) were PCR negative. On the other hand,19 out of the 30 ELISA-positive HM patients were negative by PCR. Failure to detect *Toxoplasma* DNA in a considerable number of seropositive individuals was reported in different population groups[36,37]. In Brazil, nested PCR for detection of *Toxoplasma b1* gene was negative in 90% of 353 seropositive blood donors[38]. This can be explained by the low amount of DNA in blood samples. In chronic toxoplasmosis, parasites appear rarely in the blood at intervals during the asymptomatic phases of the disease and may be detected by PCR unless efficiently eliminated by the immune system[39]. Bavand *et al.*[40] studied HIV patients in Iran and reported that *Toxoplasma* DNA was detected by PCR amplification of *529re* gene in only five out of 69 *T. gondii* IgG-positive patients. They proposed that the immunological status in these patients might not be severely depressed and could have contributed to the clearance of blood parasitaemia[40]. It has to be noted that the absence of DNA does not exclude the presence of active disease[7,41,42]. Local reactivation of latent cysts may not be associated with positive PCR results in peripheral blood samples. Molecular diagnosis is more useful in cases associated with severe localized infection or dissemination[39].

Intriguingly, the present study showed that *Toxoplasma* DNA was detected in two patients for whom the results of serological tests were negative. A possible explanation is that blood samples were drawn very early after infection and before the production of antibodies. Another explanation is that the immune system was unable to produce enough immunoglobulins to be detected by the serological ELISA assay due to immunodeficiency. That is why parasite detection by PCR is strongly recommended when serology is negative in immunocompromised patients, with PCR offering high sensitivity in these settings. Collectively, PCR and serological examination showed slight agreement. Therefore, the combination of both techniques would be more accurate for a definite diagnosis of toxoplasmosis.

Neither IgG nor IgM seropositivity was significantly associated with positive PCR results among HM patients. While PCR can detect parasites that have been released from tissue cysts into the bloodstream[43], it cannot differentiate the DNA of dead and viable parasites. The parasite may be rapidly destroyed by the immune system but the DNA remains in the blood for up to 13 w[44]. Therefore, molecular detection may overestimate the presence of the parasite in peripheral blood[38]. Diagnosis of *Toxoplasma*-related illness in immunocompromised patients should be made using a combination of molecular, clinical, and radiologic findings[42].

In the present study, the seropositivity of *T. gondii* showed a non-statistically significant association with the age and gender of patients. Similar results were reported in previous studies[17,21,45]. On the contrary, Tektook *et al.*[46] reported a significant increase in *T. gondii* seroprevalence with the age of prostatic cancer patients and attributed this to the fact that older patients are more likely to have been exposed to infection than younger ones[46]. The discrepancy between studies may be related to the socioeconomic level of the population where the infection is acquired at a relatively younger age in populations of the lower socioeconomic class compared to the higher class[7]. Contact with infected cats and consumption of undercooked meat are the main sources of *T. gondii* infection in man[47]. However, both were not significantly associated with *T. gondii* infection in HM patients. These findings correlate with the findings of an earlier report among different population groups in Egypt[48]. Oocysts are not usually found on cat fur but are often buried in soil along with cat stool. Soil contaminated by the infected cats stool, is the main source of human infection. Sanitation and hygienic habits therefore play a significant role in limiting the transmission of oocysts to man[49]. Owned cats vaccination against *T. gondii* could reduce the number of oocysts in the environment[50]. Transmission through undercooked meat is largely determined by the prevalence of the parasite in the animals as well as local methods of meat cooking[51].

Although immunodeficiency is a common side-effect of treatment in HM patients[2], the present study demonstrated that patients who had started chemotherapy were not at higher risk of toxoplasmosis. This may be attributed to the fact that drugs that interfere with tumor proliferation possess considerable anti-*Toxoplasma* activity[52]. Blood transfusion is a potential transmission route for toxoplasmosis that can lead to serious clinical consequences in immunocompromised patients[53,54]. In the present study, blood transfusion was not linked to *T. gondii* transmission in HM patients. Transmission of *Toxoplasma* by blood transfusion depends on the level of parasitemia in blood donors. *Toxoplasma* parasitemia was previously reported in 10% of blood donors in Egypt which suggests that the rate of *Toxoplasma* transmission is low[15].

In conclusion, HM is a predisposing factor for toxoplasmosis with high risk of reactivation. The combination of serology and PCR is more accurate to reach a definite diagnosis. Follow-up is necessary to prevent the development of life-threatening disease in these patients. Further studies should investigate the effects of disease duration, length, and type of anti-cancer therapy and the severity of immune suppression on the outcome of *T. gondii* infection in HM patients.

Author contribution: El-Taweel HA proposed the study topic, and Khalil SS designed the study protocol. El-Gammal MA supervised enrollment of patients and participated in data collection and presentation. Shawa TM carried out data collection and laboratory tests. Ibrahim HS performed laboratory tests, and interpreted data. Shawa TM and Ibrahim HS wrote the manuscript. El-Taweel HA and Khalil SS revised the final version of the manuscript. All authors accepted the authorships and the final version of the manuscript before publication.

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