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Original paper

Study of toxoplasmosis in type 2 diabetic patients using ELISA and B1 nested-PCR methods

Jasem SAKI[1], Iman KHODKAR[2,3], Farnoosh SHADNOOSH[2], Mahmoud SAFI[2], Ataallah GHADIRI[4], Shahla SHAFIEENIA[2]

[1]Health Research Institute, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Department of Medical Parasitology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[4]Department of Immunology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Corresponding Author: Jasem Saki; e-mail: jasem.saki@gmail.com

ABSTRACT. *Toxoplasma gondii* (*T. gondii*) is a ubiquitous, opportunistic organism, which actually infects all warm-blooded animals. Diabetes is a silent, irritating metabolic disorder among which type 2 diabetes includes over 90% of cases globally. This case-control study was aimed to detect *T. gondii* infection in type 2 diabetic patients in Khuzestan province, southwest of Iran. Serological enzyme-linked immunosorbent assay (ELISA) and molecular polymerase chain reaction (PCR) method targeting B1 gene were employed to comparatively detect the parasitic infection among 377 diabetic patients and 200 non-diabetic subjects during 2016–2018. Considerably higher anti-*T. gondii* antibodies were determined in case group 44.29% (167/377), than in control group 19% (38/200) (P<0.05). Among diabetic patients, 153 (40.58%) were seropositive for IgG and 14 (3.71%) were seropositive for IgM, while 35 (17.5%) and 3 (1.5%) healthy people were seropositive regarding IgG and IgM, respectively (P<0.05). By nested PCR, B1 gene was identified in 36 out of 167 (21.55%) and 5 out of 38 (13.15%) of the seropositive samples of case and control groups, respectively. The prevalence of anti-*T. gondii* antibodies and DNA in diabetic patients was significantly higher than in non-diabetic patients. Thus, it could be recommended to routinely evaluate the chronicity of the infection in diabetic patients.

Keywords: toxoplasmosis, type 2 diabetes, ELISA, nested PCR

Introduction

The model apicomplexan, *Toxoplasma gondii* (*T. gondii*), is an obligatory intracellular parasite that prevails the world by infecting almost all warm-blooded animals including humans; in this sense, cats play a major role as definitive hosts. The parasite possesses different infective stages to guarantee its transmission, including bradyzoites (raw/under-cooked livestock meat), tachyzoites (transplacental /trans-mammary transmission) and oocyst-embedded sporozoites (contaminated food/water sources) [1]. Toxoplasmosis is mostly asymptomatic in those individuals with healthy immune status, whereas it represents as chorioretinitis, brain calcification and fetal abortion in pregnant women or pneumonitis as well as encephalitis in immunocompromised patients [2–5]. The worldwide prevalence of *T. gondii* infection varies between 10% and 80% depending on the geographical region [6–10]. In Iran, western Asia, the prevalence of the parasitic disease seems to be high, reaching over 50% in some regions [11,12]. Based on the published literature, the prevalence of *T. gondii* infection in Khuzestan province, southwest of Iran, ranges between 21.5 to 47.25 % [13–18]. Diabetes mellitus (DM), is a silent but devastating metabolic disorder characterized by a high blood glucose level over long periods. It is noteworthy that type 2 diabetes includes over 90% of DM cases globally [19]. For example, in the United States the prevalence of DM (diagnosed and undiagnosed patients) was estimated to be about 9.3% and there

were, also, reports indicating 26% of the community were in the pre-diabetic condition [20].

Two major forms with distinct etiologies have been recognized for diabetes mellitus: type 1 and type 2. The disease is very similar to immunodeficiency disorders like Human Immunodeficiency Virus (HIV) infection, so that it impacts innate immunity, leading to a higher degree of susceptibility to opportunistic infections. Previously, some studies suggested that opportunistic infections such as *T. gondii* are more common in diabetic patients and timely diagnosis and detection of toxoplasmosis in at-risk patients is a necessity to prevent disease propagation [21–24].

Various serological test have been developed and used to detect anti-*Toxoplasma* antibodies, comprising enzyme-linked immunosorbent assay (ELISA), indirect fluorescence antibody (IFA), the latex agglutination test and haemagglutination tests [25]. Molecular tools are more preferred in epidemiological studies for accurate detection of *T. gondii* DNA in different clinical samples. In this sense, polymerase chain reaction (PCR)-based methods are of utmost interest for molecular diagnosis [26,27]. The nested-PCR technique has been shown to possess reliable sensitivity for diagnosing *T. gondii* infection, which renders it as one of the most significant assays in epidemiological studies [28]. In Fallahi et al. [29] study, this method showed adequate sensitivity and specificity for detecting toxoplasmosis. On this basis, here we utilized two reliable methods, ELISA and nested PCR, to detect IgG and IgM anti-*Toxoplasma* antibodies as well as *T. gondii* genomic DNA in diabetic and non-diabetic people of Ahvaz County, southwest of Iran.

Materials and Methods

This research project was registered at the Ahvaz Jundishapur University of Medical Sciences under OG 93144 code. All methodology has been approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences. At first step, a consent was obtained from the participants.

In the present study ELISA was used to detect specific anti-*T. gondii* IgG and IgM antibodies in type 2 diabetic patients' sera. In the following, a nested-PCR assay was carried out to target *Toxoplasma* B1 gene and determine acute toxoplasmosis in diabetic and non-diabetic patients in Khuzestan province, south west of Iran.

Patients and blood samples

In current study, the blood samples were collected from 377 clinically-confirmed type 2 diabetic patients in Training Affiliated Hospitals of Ahvaz Jundishapur University of Medical Sciences during 2016–2018. In addition, 200 serum samples were gathered from non-diabetic individuals as control group. Sera and buffy coat samples were separated and stored in –20°C until further use. In this study, diabetic patients were sorted into three groups regarding the the duration of DM: group 1 with short-duration (0–2 years), group 2 with moderate duration (2–4 years), and group 3 with long duration (> 4 years).

Immunological methods

ELISA test

Determination of IgM and IgG anti-*Toxoplasma* antibodies in participants' sera was performed using NovaLisa® *Toxoplasma gondii* kit (GenWay Biotech Inc., Germany).

IgG avidity test

To distinguish between acute and chronic phases of the infection, an ELISA-based *Toxoplasma gondii* IgG avidity test was done and interpreted based on the kit instructions (Toxo-IgG-avidity kit; TEC Immundiagnostica GmbH, Germany).

Molecular tests

Nested PCR

Molecular assay was done based on the extracted genomic DNA from buffy coat samples using DNeasy tissue kit (Bioneer, Korea), based on the manufacturer's instructions. In the next step, genomic DNA was resolved in 50 µl elution buffer and stored at –20°C to be further used. The B1 gene of *T. gondii* was detected by nested PCR and the experimental procedure was done according to a previously described method [30–32]. The amplified PCR products were separated on a 2% (W/V) agarose gel containing DNA safe stain and visualized under ultraviolet (UV) illumination of a GelDoc apparatus.

Data analysis

The gathered information reported here was analyzed using SPSS software (version 18). The Chi-square (χ^2) and Fisher's exact tests were used to compare the seroprevalence values, related to the demographic characteristics of the subjects. $P<0.05$ was considered as the level of significance.

Table 1. Seroprevalence of *T. gondii* infection in healthy and diabetic groups

Group	No. of tested samples	Seropositive	IgG^+	IgM^+	High IgG avidity (chronic)	Low IgG avidity (acute)
Healthy controls	200	38(19%)	35(17.5%)	3(1.5%)	30/35(85.71%)	5/35(14.28%)
Diabetes cases	377	167(44.29%)	153(40.58%)	14(3.71%)	142/153(92.81%)	10/153(6.53%)
P-value	–	<0.05	<0.05	<0.05	<0.05	<0.05

Results

IgG and IgM ELISA assay

In total, 377 and 200 sera from diabetic type 2 patients and healthy individuals were serologically and molecularly analyzed in the present study regarding *T. gondii* infection. The overall seroprevalence of *T. gondii* infection was estimated to be 44.29% (167/377) and 19% (38/200) for case and control groups, respectively ($P<0.05$). Among DM cases, 153 (40.58%) were seropositive for IgG and 14 (3.71%) were seropositive regarding IgM. Of control group, 35 (17.5%) were seropositive for IgG, while only 3 (1.5%) showed to be seropositive for IgM, indicating statistically significant findings ($P<0.05$). In order to exactly differentiate acute and chronic phases of *T. gondii* infection, IgG avidity ELISA test was done on 153 and 35 IgG positive samples reported from case and control groups, respectively. In this sense, 142/153 (92.81%) DM patients indicated high IgG avidity, suggesting past infection, whereas 10/153 (6.53%) DM cases had low IgG avidity, representing recent infection. Likewise, 30/35 (85.71%) and 5/35 (14.28%) non-diabetic individuals had high and low IgG avidity indices, respectively (Tab. 1). With respect to the duration of DM and its possible association with toxoplasmosis, it was shown that the prevalence of toxoplasmosis was directly correlated with the duration of DM, so that the highest rate of *T. gondii* infection [89/170 (52.35%)] was detected in group 3 having long DM duration (>4 years) (Tab. 2).

Table 2. Comparison of the *T. gondii* infection prevalence in diabetic patients in terms of disease duration

Duration of diabetic (year)	No	$ELISA^+$	PCR^+
0–2	97	28(28.86%)	2/28(7.14%)
2–4	110	50(45.45%)	8/50(16%)
>4	170	89(52.35%)	26/89(29.21%)
Total	377	167(44.29 %)	36/167(21.55%)
P-value	–	<0.05	<0.05

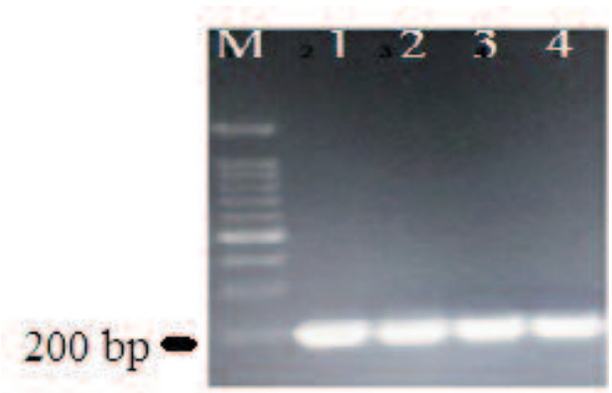

Figure 1. Gel electrophoresis of PCR product of *T. gondii* from buffy coat samples. Lane M: 100 bp ladder DNA size marker; Lane1: positive control; Lane 2–4: positive samples

Nested PCR

Nested-PCR was performed on 205 (167=case and 38=control) seropositive sera specimen. According to our results, B1 gene of *T. gondii* was amplified in 36/167 (21.55%) samples from DM patients and 5/38 (13.15%) specimens from healthy people. Our findings showed that the higher is the duration of the diabetes, the higher is the chance of identification of the parasitic DNA in the patients' blood (Tab. 2 and Fig. 1).

Discussion

Diabetes mellitus is a metabolic disorder caused either by insufficient production of insulin by pancreas or improper cellular response to the produced insulin hormone. Reportedly, DM has been known as one of the main emerging health threats, and by 2025, approximated DM cases would reach to 380 million individuals globally

[33]. It is estimated that over 90% of DM cases worldwide have type 2 diabetes (T2D), which represents a rising epidemic [34].

Some of the immunological imbalances of diabetes have been previously highlighted in studies on DM patients, including dysregulation of innate immunity, reduced T-cell response and neutrophil function as well as humoral disturbances [34–36]. Consequently, these patients may become susceptible to opportunistic infections such as *T. gondii* infection. Therefore, timely detection of *T. gondii* in at-risk people can prevent disease progression and its sequelae. The present seromolecular investigation clarified that out of 377 diabetic patients, 40.58% were regarded as seropositive for the *T. gondii* IgG antibodies, while 17.5% out of 200 individuals were found to be seropositive among healthy controls. These results are in agreement with previous studies that reported higher anti-*T. gondii* antibodies in diabetic patients, than in nondiabetic fellows [37].

Herein, we reported a seroprevalence of 44.29% (167/377) in examined diabetic patients in Ahvaz. The obtained results were lower than Shirbazou et al. [21], Jafari Modrek et al. [22] and Gocke et al. [38] studies that reported seroprevalence rates of 60%, 70.3% and 56% in diabetic patients, respectively. Various factors such as nutrition style, contact with dog/cat, consumption of raw meat, duration of diabetes, severity of non-infectious complications and likely co-infections could substantially impact the prevalence of *T. gondii* infection [39]. The results of the present study indicated 3.71% of diabetic cases had positive IgM antibody, being relatively close to the prevalence rate reported by Saki et al. [31] on cancerous children (6.4%), Saki et al. [40] on diabetic pregnant women (2.7 %) and Hemida et al. [41] in diabetic patients (2%). As shown in table 1, the number of cases with low avidity in our research was only 6.5%, near to the avidity reported by Abolghasem et al. [42] (7.1%) on pregnant women.

In clinical circumstances, serological tests such as IFA and ELISA are routinely employed to detect specific IgM- and IgG antibodies. Evidently, performing serological assessments are not reliable by themselves for detection acute *Toxoplasma* infection and further clinical inference should accompany with complimentary avidity and molecular tests [41]. Utilization of more sensitive and specific techniques such as PCR has been shown to be more effective [43]. Moreover, the eligibility of PCR to diagnose active form of toxoplasmosis is of most importance for immunocompromised cases, particularly when serological tests fail to diagnose. Most of the molecular investigations have used B1 gene for the detection of *Toxoplasma* [30,31,44]. In current study, seropositive individuals for specific anti-*Toxoplasma* IgG and/or IgM antibodies were further evaluated to detect *T. gondii* DNA in buffy coat samples.

In this study, B1 gene was successfully amplified in 21.55% of 167 seropositive diabetic patients and in 13.15% of 38 seropositive healthy control, which was statistically significant. Such a molecular prevalence reported here was significantly lower in comparison with Mousavi et al. [44] study in Zahedan, eastern Iran (56.60%) which used a nested-PCR assay targeting B1 and RE genes of *T. gondii*.

In this survey, there observed no harmony between serology and PCR results, since only 36/167 (21.55 %) of examined seropositive samples were positive by nested-PCR. There are two possible explanations: first is that the clearance time for *Toxoplasma* DNA from the patient's blood (5.5–13 weeks) [45] indicates an acute infection that is likely to be clinically important. "Conversely, a small number of parasites might have been released from tissue into the blood at a subclinical level and their presence can only be detected by accurate methods such as PCR" [46]. Second, is defects in immune responses in diabetic patients. A number of imbalances such as low complement factor 4 and decrease in cytokine response upon stimulation have been reported in humoral and innate immunity in diabetic patients [47].

In this study we found positive relevance between *T. gondii* infection and duration of diabetes. There are some explanations for such a possible association; recently, some studies have highlighted the probable relevance of DM and infectious agents like *Helicobacter pylori* [48] and Coxsakie B4 virus [29,49]. In this regard, the apicomplexan parasite, *T. gondii*, has been proposed as a likely cause of diabetes, and current knowledge derived from the scientific literature nearly rely on this association [1,23,37,50,51]. In a meta-analysis on case-control studies, Majidiani et al. [52] suggested chronic toxoplasmosis as a possible risk factor for type 2 DM. Of note, the authors found no statistically significant relevance between *T. gondii* and type 1 DM.

Based on a new experimental study, the concentration of insulin can remarkably increase the intracellular replication of *T. gondii* tachyzoites [53]. As well, immunodeficiency status in diabetic patients may provide a basis for the onset of toxoplasmosis. In these cases, latent *Toxoplasma* tissue cysts would revive and rupture, leading to the high rate of tissue invasion and severe brain damages [54].

In conclusion, this study showed that the prevalence of *T. gondii* infection among diabetic patients is higher than that of healthy people (P<0.05) and the prevalence directly associates with the duration of diabetes.

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