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RESEARCH ARTICLE

Prevalence of Toxoplasmosis among Aborted Women and Immunohistochemical Evaluation of their Placenta

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Abstract

Toxoplasmosis is one of the zoonotic diseases caused by the obligate intracellular protozoan *Toxoplasma gondii*, which is associated with spontaneous abortion in infected pregnant women. In the present study, the sera of 228 first-trimester aborted women were screened for anti-*Toxoplasma* IgG and IgM antibodies, and the infection was further assessed in the blood and placenta of positive and negative cases by molecular methods. The results revealed that 47.4% of aborted women were sero-positive for toxoplasmosis (AT) by the latex agglutination test, while 52.6% were negative (NT). It was also found that 74.1% of AT cases were positive for IgG, while 25.9% were positive for IgM. In addition, the examined AT cases showed the amplified DNA fragment of the parasite, while it was not observed in NT samples. The placenta of molecularly diagnosed AT and NT cases were subjected for immunohistochemical expression evaluation for T-bet, GATA-3, FOXP3, IL-17, CD8, CD68 and perforin. A significant increased expression of T-bet, IL-17, CD8, CD68 and perforin was observed in AT cases compared to NT cases. In contrast, FOXP3 showed a significant decreased expression in AT cases, while no significant difference was observed for GATA-3. In conclusion, toxoplasmosis is an important risk factor for spontaneous abortion, which was associated with abnormal expression of some immunological markers in placenta.

Keywords: *Toxoplasmosis, Abortion, Prevalence, Immunohistochemical expression.*

Introduction

Toxoplasmosis is one of the zoonotic diseases caused by an obligate intracellular protozoan (*Toxoplasma gondii*) belongs the Phylum Apicomplexa. It is most commonly transmitted to human from food or water contaminated with the parasite tissue cysts or oocysts; however, a congenital transmission from a pregnant mother to her fetus (vertical transmission) can also occur at a rate of approximately 20%, as reported by a study based on a PCR analysis of the parasite genome [1].

During the infection, both innate and acquired immune responses are initiated to control toxoplasmosis and the spread of parasite, in which cellular interactions occur and initially involve cells of the innate immune system that promote for an effective acquired immune response.

The latter is mainly mediated by T helper 1 (Th1) cells via two important cytokines; interferon-gamma (IFN-γ) and interleukin-12 (IL-12), which are effective in the resistance against *T. gondii* [2]. For innate immune system, enterocytes, monocytes, dendritic cells (DCs), macrophages, natural killer (NK) cells and neutrophils are non-specifically activated by *T. gondii* in order to limit the proliferation of parasite and initiate the adaptive immune response [3].

However, parasite is recognized by its ability to cross biological barriers (placenta, brain and eye) and infects cells in these sites. In placenta, *T. gondii* infects trophoblasts, which are important cells for the growing fetus, as they mediate the exchange of gases and nutrients at the maternal / fetus surface [4].

In the case of normal pregnancy, different immune factors contribute its success and persistence, but if they are dysregulated a spontaneous abortion can occur; for instance, abnormal expression of some human leukocyte antigens (HLA) and imbalance between Th1 and Th2 lymphocytes, as well as abnormalities in the function of the natural killer (NK) cells [5]. Cytokines, the degree of expression of adhesion molecules and some other cellular immunological markers are further immunological factors that play an important role in the success of pregnancy, and their disruption may also lead to spontaneous abortion [6].

Toxoplasmosis is one of the most important factors involved in the dysregulation of the forthcoming immunological factors in pregnant women, especially during the first three months of pregnancy. In this context, studies have suggested that cytokines (IL-2, IL-12, IL-4, IL-10, IL-17A and TNF-α) are involved during the course of pregnancy in infected women [7].

These factors are produced by important immune cells, such as Th1, Th2 and Th17 cells, as well as T regulatory (Treg) cells, macrophages, and NK cells, in which the cellular polarization between them plays an important role in the success of pregnancy, whether in normal pregnancy or in the case of toxoplasmosis [8].

These cells are distinguished by their immunohistochemical (IHC) markers, such as T-bet, GATA-3, FOXP3, IL-17, CD8, CD68 and perforin (9, 10). Their expression in placental tissue has been demonstrated, and it has been suggested that their aberrant expression may risk the pregnancy, especially in pregnant women infected with toxoplasmosis [11, 12].The present study was planned to estimate the prevalence of toxoplasmosis among aborted women by serological and molecular methods. In addition, the IHC expression of T-bet, GATA-3, FOXP3, IL-17, CD8, CD68 and perforin was inspected in the placental tissue of toxoplasma-positive and –negative cases.

Materials and Methods

Sample Investigated

The study was approved by the Ethics Committee at the Iraqi Ministry of Health. A prospective cross-sectional hospital based-study was carried out during the period March-December, 2015, in which 228 first-trimester pregnant women were admitted to the Educational Gynecological Hospital in Karbala (a city located 100 km south-west the capital Baghdad) due to a threaten pregnancy. The last menstrual period was considered to determine the gestational age, which was confirmed by ultrasound scan examination. Before the curettage, venous blood was collected and distributed into plain and EDTA tubes. After curettage, placental biopsies were also obtained and kept in 10% formalin until isolation of DNA.

Serological Detection of Toxoplasmosis

The sera were separated from collected blood by centrifugation (3000 rpm for 10 minute), and then kept in sterile Eppendorf tubes at -20°C until use for serological assessments. The sera of all participating aborted women were first screened for anti-*Toxoplasma* antibodies by the latex agglutination test (Toxo-Latex kit, Spinreact, Spain). Sera of positive cases were further assessed for anti-*Toxoplasma* IgG and IgM antibodies by enzyme linked immunosorbent assay (ELISA) test kits (*Toxoplasma* IgG and IgM kits, ACON, USA). In both types of assessments, the instructions of manufacture were followed.

Molecular Detection of Toxoplasmosis

The detection of *T. gondii* DNA was carried out in DNA isolated from EDTA blood and placental biopsies. The blood DNA was isolated using ReliaPrep™ Blood gDNA Miniprep System (Promega, USA), while for placental DNA, the g SYNC™ DNA extraction kit (Gene aid, Thailand) was used. The DNA samples were subjected to PCR amplification of *T. gondii B1* gene by a previously published method [13]. The reaction mix (25 µl) consisted of 12.5 µl GoTaq® green master mix (Pioneer-Korea), 1 µl forward primer (5'-GG AA CT GC AT CC GT TC AT GAG-3'), 1 µl reverse primer (5'-TC TT TA AA GC GT TC GTGGTC-3'), 5 µl DNA and 5.5 µl nuclease-free distilled water.

The PCR amplification conditions were initial denaturation at 95ºC for 1 minute (1 cycle), followed by 35 cycles of denaturation at 95ºC (35 seconds), annealing at 55ºC (15 seconds) and extension at 72ºC (30 seconds). A further cycle of a final extension at 72ºC (7 minutes) was also applied. The PCR products were electrophoresed on 2% agarose containing Sybr green stain, and the bands were

visualized using UV gel-documentation system.

Immunohistochemical (IHC) Expression Assay

The IHC expression of T-bet, GATA-3, FOXP3, IL-17, CD8, CD68 and perforin was inspected in placental tissue sections by the labeled streptavidin-biotin method [14]. The method depends on a primary antibody (ABCAM, USA) interaction with the target antigen in tissue. Then, a biotinylated secondary antibody (DAKO, Denmark) is added to react with the primary antibody. After that, peroxidase-conjugated streptavidin is added to form a complex with the secondary antibody (DAKO, Denmark).

Finally, a chromogen solution containing diamino benzidine (DAKO, Denmark) is added and results in the formation of a colored precipitate in the site of target marker. Ten randomly selected fields were examined microscopically, and the number of positive and negative cells was recorded. Percentage of positive cells out of total examined cell was considered as score for each sample [15].

Statistical Analysis

The data were given as either number and percentage frequencies or mean ± standard error of mean (SEM).

In the former case, Pearson Chi-square test (X^2) was employed to assess significant differences, while in the latter case, analysis of variance (ANOVA) was used. A probability value (p-value) equals or less than 0.05 was considered significant. The SPSS package version 13.0 was employed to carry out these analyses.

Results

Out of the 228 aborted women, 108 (47.4%) were sero-positive for toxoplasmosis (abortion due to toxoplasmosis; AT) by the latex agglutination test and their age range was 16 - 42 years, while 52.6% were negative (age range: 16 - 43 years). The negative cases were considered as a control group, in which the cause of abortion is not toxoplasmosis (NT).

The sera of AT group were examined for anti-*Toxoplasma* IgG and IgM antibodies. It was found that 74.1% of cases were positive for IgG, while 25.9% were positive for IgM. Age distribution of these cases (16 - 25, 25 - 35 and 36 - 42 years) revealed that 36.2, 33.8 and 30.0% of IgG positive case were respectively positive. The corresponding percentages for IgM were 39.3, 32.1 and 28.6%, respectively. However, there were no significant variations between age groups in the distribution of IgG or IgM positive cases (Table 1).

Table 1: Number and percentage frequencies of anti-*Toxoplasma* IgG and IgM antibody-positive cases

Groups	Anti-*Toxoplasma* Antibody-Positive Cases			
	IgG		IgM	
	N	%	N	%
Total (N = 108)	80	74.1	28	25.9
Age Groups (years)				
16 - 25	29	36.2	11	39.3
26 - 35	27	33.8	9	32.1
36 - 42	24	30.0	8	28.6
p-value	NS		NS	

NS: Not significant (p-value > 0.05)

Twenty-five of AT IgG-positive group and a similar number of NT group were selected for a further assessment of toxoplasmosis by PCR amplification of *T. gondii B1* gene. All

members of AT group showed a single band that had a molecular size of 193bp after agarose-gel electrophoresis of DNA isolated from blood and placenta, while none of the NT samples showed such band (Figure 1).

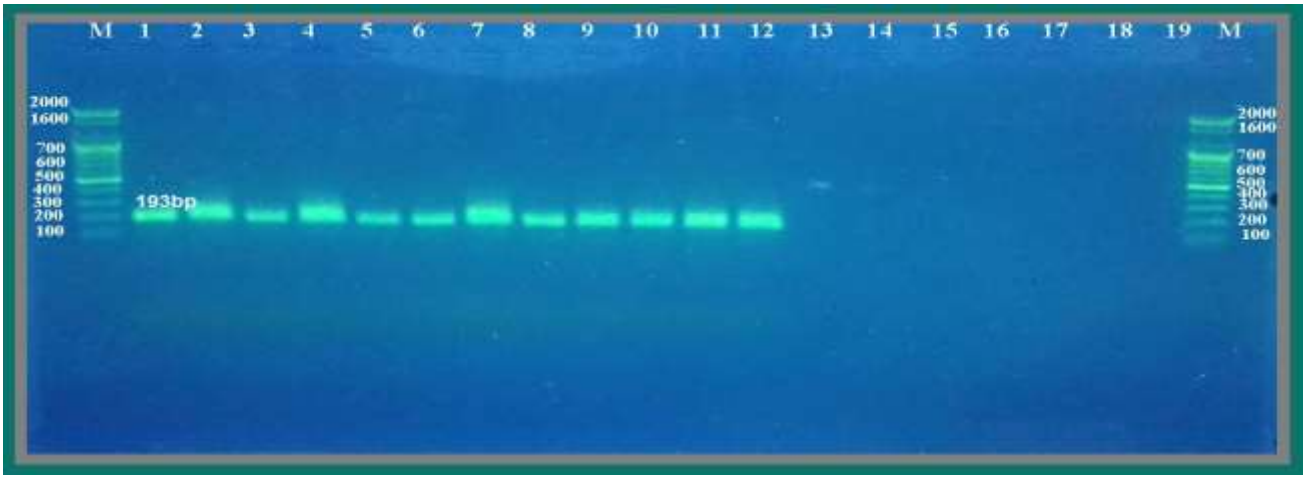

Figure 1: Agarose-gel (2%) electrophoresis after PCR amplification of *T. gondii B1* gene from blood and placenta showing a band of 193bp. Lane M: DNA ladder (100bp); Lanes 1 - 6: blood samples of toxoplasmosis-aborted women; Lanes: 7 - 12: placenta samples of toxoplasmosis-aborted women; Lanes 13 - 15: blood samples of non-toxoplasmosis aborted women; Lanes 16-19: placenta samples of non-toxoplasmosis aborted women

The placenta of PCR-examined AT and NT cases were subjected for IHC assessments to evaluate T-bet, GATA-3, FOXP3, IL-17, CD8, CD68 and perforin expression (Figure 2). A significant increased expression of T-bet (36.2 ± 13.2 *vs*. 8.3 ± 4.3%; $p \leq 0.01$), IL-17 (24.9 ± 7.1 *vs*. 4.4 ± 1.2%; $p \leq 0.01$), CD8 (19.3 ± 5.3 *vs*. 3.8 ± 1.4%; $p \leq 0.01$), CD68 (32.2 ± 6.3 *vs*. 15.0 ± 3.9%; $p \leq 0.01$) and perforin (15.2 ± 5.4 *vs*. 3.2 ± 1.1%; $p \leq 0.01$) was observed in AT cases compared to NT cases. In contrast, FOXP3 showed a significant decreased expression in AT cases (10.3 ± 3.6 *vs*. 23.6 ± 6.5%; $p \leq 0.01$), while no significant difference was observed for GATA-3 (22.2 ± 8.3 *vs*. 17.9 ± 8.9%; $p > 0.05$) (Table 2).

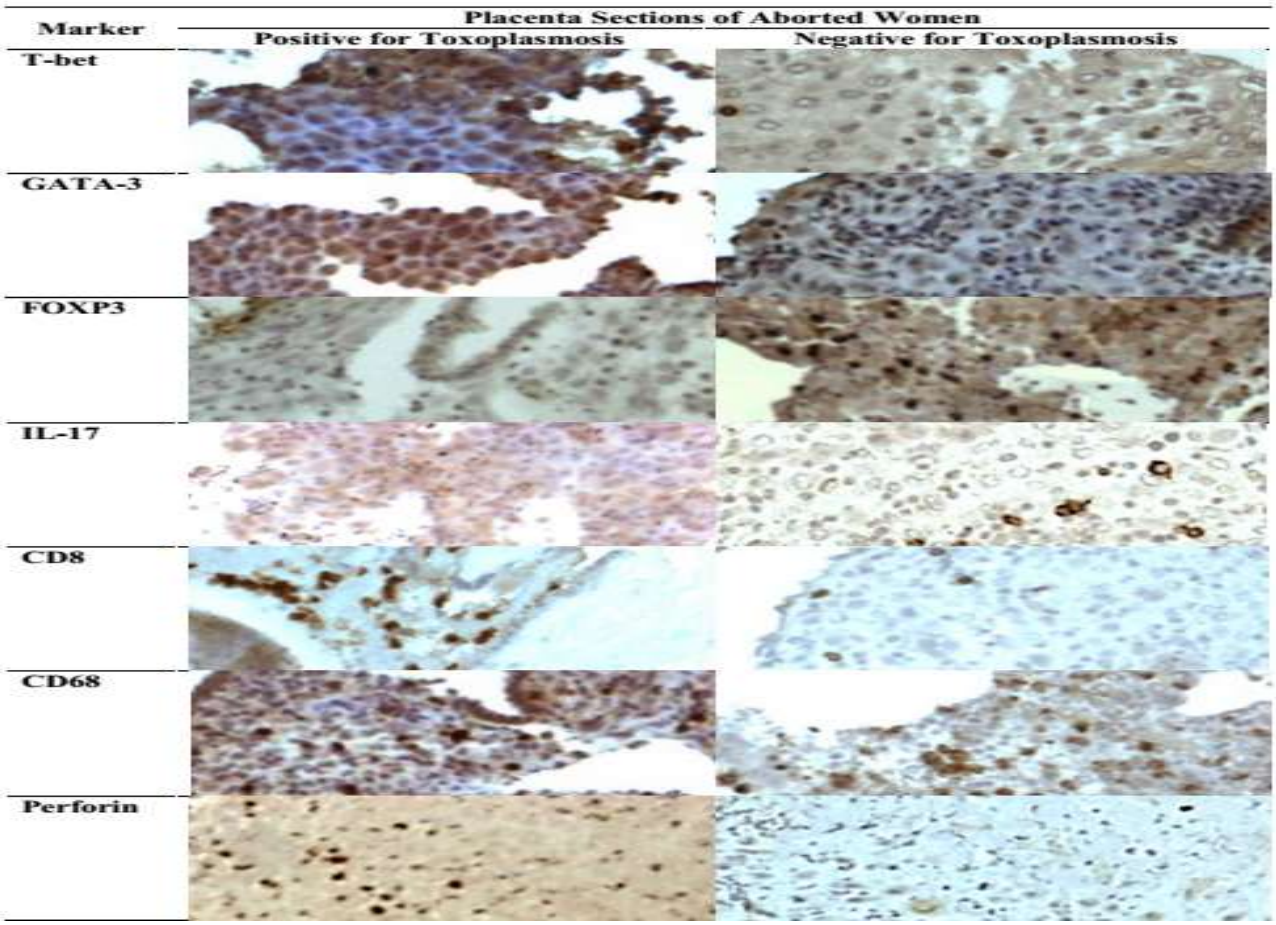

Figure 2: Cross-sections of placenta showing immunohistochemical expression of T-bet, GATA-3, FOXP3, IL-17, CD8, CD68 and perforin (40X) of toxoplasmosis-positive and –negative aborted women

Table 2: Percentage frequency of T-bet, GATA-3, FOXP3, IL-17, CD8, CD68 and perforrin immunohistochemical expression in placenta of toxoplasmosis and non-toxoplasmosis-aborted women

Marker	Immunohistochemical Expression (%; Mean ± SEM)		*p*-value ≤
	Toxoplasmosis-Aborted Women (No. = 25)	Non-toxoplasmosis-Aborted Women (No. = 25)	
T-bet	36.2 ± 13.2	8.3 ± 4.3	0.01
GATA-3	22.2 ± 8.3	17.9 ± 8.9	NS
FOXP3	10.3 ± 3.6	23.6 ± 6.5	0.01
IL17	24.9 ± 7.1	4.4 ± 1.2	0.01
CD8	19.3 ± 5.3	3.8 ± 1.4	0.01
CD68	32.2 ± 6.3	15.0 ± 3.9	0.01
Perforin	15.2 ± 5.4	3.2 ± 1.1	0.01

NS: Not significant

Discussion

The prevalence of toxoplasmosis in the present aborted women was 47.4%, which was higher than a previously reported prevalence (21.5%) in aborted women from Baghdad [16]. However, a higher prevalence (60.7%) was also reported in Egyptian aborted cases, while in Chinese, European and American cases, a much lower prevalence (less than 20.0%) was reported [17]. Such differences were probably related to the general hygiene parameters applied in these populations.

In addition, the incidence of *T. gondii* in the definitive host population (cats) is a further important risk factor; as such incidence shows different profiles in countries around the globe In the present study, the IHC expression of T-bet was significantly increased in placenta tissue of AT patients compared to NT cases; an observation that suggests an association between this marker and toxoplasmosis and/or abortion.

T-bet is described as extremely an important immunological factor for the differentiation of naïve T cells to the Th1 lymphocytes, and its increased expression leads to the dominance of latter cells and enhances their responsiveness in the uterine environment; an outcome that may have the potential to risk the pregnancy [18]. In addition, T-bet is also associated with an increased expression of IFN-γ to control toxoplasmosis, and thus Th1 and NK cells are increased in terms of production and function [19].

Such immunological changes in the placenta, especially of AT cases, are suggested to increase the risk of miscarriage [20]. The increased expression of T-bet was also associated with up-regulated expressions of CD8, CD68 and perforin, which are immunological markers that are associated with an increased cytotoxicity and threatened pregnancy. CD+8 and CD+68 cells and perforin are reported to be enhanced to control the parasite *T. gondii* and prevent its spread, but in turn, they also disturb the immunological equilibrium of placenta and put pregnancy under the risk of abortion [21].Such increased expressions were encountered by a decreased expression of FOXP3, which is a marker for important cells that are involved in regulation of immune response in placenta of normal and AT cases [22].

The importance of this factor lies in the development and function of natural CD4+CD25+Treg cells, and its ability to control the production of some pro-inflammatory cytokines such as IL-12 and TNF-α [23, 25]. Such findings confirm a previous study, in which a decrease in FOXP3+ cells was reported in the placenta of mice experimentally infected with toxoplasmosis, and such decrease was associated with the induction of apoptosis and fetal loss [26]. In addition, Lu and colleagues [27] reported decreasing cell numbers of Treg cells in the spleen and at the separation surface between mother and fetus of infected mice.

It was found that the parasite induced apoptosis in placenta cells, and consequently high rates of abortion, bleeding and low fetal weights were observed in infected mice. IL-17 was a further marker associated with toxoplasmosis and abortion, and the present results depicted a significant increased expression of this marker in AT patients compared to NT cases.

A previous investigation linked IL-17 to abortion, and the authors reported a high

proportion of cells positive for this marker in the fallen tissue of unexplained repeated spontaneous abortions compared to those with normal pregnancy [28]. Such cellular increase in placenta tissue, especially during the first trimester of pregnancy may threaten the life of fetus, where a balance between the Th17 and Treg cells is required to perpetuate the pregnancy [30]. A further study noticed that toxoplasmosis disrupt the natural balance between the Th17 and Treg cells in the spleen and the placenta of infected mice, and a decrease in the proportion of Treg cells was recorded in the tissue of placenta [28]. Further studies confirmed the dominance of Th17 cells in a number of pregnancy disorders; such as pre-term birth and pre-eclampsia, as well as abortion due to toxoplasmosis [31, 32].In conclusion, toxoplasmosis is an important risk factor for spontaneous abortion, which was associated with abnormal IHC expression of some immunological markers in placenta. In this context, T-bet, CD8, CD68, perforin and IL-17 were up-regulated, while FOXP3 was down-regulated. Therefore, further investigations are certainly required to determine these expressions at the RNA level to understand the molecular pathway of their abnormal expression.

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