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Toxoplasmosis in Iranian Abortion Cases with Appendectomy

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Abstract

Background: The PCR-based techniques detect *Toxoplasma gondii* DNA in patients' samples. Real-time PCR is used mainly to quantify infection agents especially in immunecompromised cases. The aim of our study was to examine the probable association of toxoplasmosis and appendectomy in an Iranian sample of women undergone a recent abortion by the performance of real-time PCR.

Methods: Sixty five abortion cases were occurred from March 2008 to October 2009 in Isfahan, Iran. Specimens from their products of conception were screened for *T. gondii* DNA by both the conventional PCR and real-time PCR. The acquired data was assessed in relation to the history of appendices by the statistical methods in SPSS software.

Results: The results of the conventional PCR were positive in 10 (15.48%) of 65 patients and negative in 55 (84.6%). After the conventional PCR, 8 (80%) PCR positive patients with surgical appendix had the average number of obtained parasites that was 10.63 which was significantly higher than the group without appendectomy ($P \leq 0.05$); that is appendectomy was associated with infection in immunosuppressive cases.

Conclusions: The pregnant women with immunocompromised condition are at a risk for toxoplasmosis and finally abortion; all *T. gondii* positive patients should be followed up with real-time PCR techniques.

Key words: *Toxoplasma gondii*, aborted pregnancy, real-time PCR, immunocompromised patients, Iran

INTRODUCTION

Toxoplasmosis is healthily relevant to congenital and postnatal infection with a worldwide distribution ranged from 11% in the United States to more than 70% in Brazil [1-2]. The organism is the third most common cause of food-borne deaths in the United States [3]. The infection is characterized by asymptomatic condition with the consequent formation of cysts that may persist latently without clinical manifestations in many organs [4-5]. The parasite is protected from attacks by the immune system during this stage. The persistent tissue cysts, most commonly located in brain, eye, heart, and skeletal muscle, may remain throughout the life of the infected host for several years [5-7].

It is particularly dangerous for pregnant woman transmitted the disease to their fetuses and newborns [6], and also for the immunocompromised hosts such as AIDS patients [8]. In severe cases, they can be affected by encephalitis, myocarditis, and chorioretintis or finally die [5,9]. Thus, detection of *T. gondii* infection with high sensitivity and specificity is crucial in the management of the disease [1]. Conventional diagnosis of acute *T. gondii* infection includes some shortcomings in test accuracy when performed in active and latent forms of disease particularly for difficult cases like AIDS patients or the immunocompromised individuals [10-12].

Polymerase Chain Reaction (PCR) minimizes the above problems and facilitates diagnosis of *T. gondii* in difficult cases. *T. gondii* PCR targets the *T. gondii* B1 gene, 35 copies of which are found in each organism [13]. Since the first identification of *Toxoplasma gondii* DNA in ocular tissue with the use of PCR in 1990 [14], many studies found that its sensitivity and specificity can cause significant pitfalls [15]. While exquisitely sensitive, PCR cannot distinguish between latent and acute *T. gondii* infection.

Therefore, the diagnosis should not rely solely on the results of a positive PCR assay [16] and utilizing a secondary detection system in concert with the initial PCR reaction leads to perfect specificity [15]. It is indicated that the combination of PCR with other diagnostic tests [17] like serologic procedures [18], enzyme-linked immunosorbent assay [ELISA], and immunoblotting [19] can improve accuracy and speed of the parasite detection. Recently, real-time PCR is applied in designing studies for deleting the limitations of conventional PCR with its rapid and accurate detection of the parasite DNA load in patient samples [20].

In literature review, the final purposes of studies administered to *T. gondii*-infected and uninfected persons have been based on the risk factors, source distribution, data gathering for planning public health interventions, etc in different populations. In present work, we performed a real time PCR in combination with conventional PCR for diagnosis and quantification of *T. gondii* DNA in Iranian pregnant women undergone abortion and examined the probable association of acquiring infection and their past appendectomy due to a immunocompromised condition.

MATERIAL AND METHODS

From March 2008 to October 2009, 65 abortion cases were happened in Isfahan, Iran. The formalin fixed, paraffin wax embedded tissue blocks were obtained from their products of conception. All patients were notified to gather further information about their age and health background by a questionnaire.

DNA Extraction

First, 5-10 µm tissue sections of formalin fixed, paraffin wax embedded tissue blocks (depending on tissue size) were transferred into 1.5-mL Eppendorf tubes for DNA isolation. To avoid cross-contamination, a new, sterile, and disposable microtome blade was used immediately before cutting each block for cleaning purposes.

Subsequently, DNA extraction was performed using the procedures according the manufacturer's protocol described for the High Pure Nucleic Acid extraction kit (Roche, Germany). First, the paraffin wax was dissolved in 300 µl of Citrisolv (xylene substitute) and was washed with ethanol to remove the Citrisolv. Cell Lysis Buffer (200 µl) and 20 µl Proteinase K Solution (20mg/ml) were then added to each sample, followed by an overnight incubation at 55°C. After the solution has cooled down to room temperature, 200µl Binding Buffer was added and the High Pure filter tube was combined with collection tube. They were centrifuged and the flow-through was discarded, then 500 µl Inhibitor Removal Buffer was added followed by centrifuging. In the next step, the DNA was washed with 500 µl Washing Buffer and eluted with the elution buffer.

Using an ultraviolet spectrometer, the absorbance of a sample of DNA solution was measured for concentration at 260 nm. The absorbance ratio at 260 and 280 nm (A260/280) was used to evaluate DNA purity. The procedure was carried out with some minor modifications, for instance, longer incubation times (overnight, approximately 16 hours) and doubling proteinase K concentration [21].

Real –Time PCR

The forward primer (TOXO-F), reverse primer (TOXO-R), and TaqMan probe was designed with the Primer Express software (PE Applied Biosystems) to specifically amplify T.gondii B1 gene for the diagnosis of toxoplasmosis by real-time PCR. The specific target DNA sequence from *T. gondii* amplified by real-time PCR was the 35-fold-repetitive B1 gene of the RH strain published [22]. Briefly, the template DNA (target gene) was added to a sample of reaction mixture containing 25 µl Universal PCR Master Mix (2X), forward primer TOXO-F (5 µl, 5 µM, 5′-TCCCCTCTGCTGGCGAAAAGT-3′), reverse primer TOXO-R (5 µl, 5 µM, 5′-AGCGTTCGTGGTCAACTA TCGATTG-3′), and TaqMan probe (5 µl, 2 µM, 6FAM-TCTGTGCAACTTTGGTGTATTCGCAG-TAMRA) to bring the final volume to 50 µl. The PCRs were carried out in a GenAmp 5700 Sequence Detection System (PE Applied Biosystems, USA). PCR amplification protocol was as follows: initial AmpliTaq Gold DNA Polymerase activation at 95 ºC (10 min), followed by 40 PCR cycles of 95°C (15 s) and 60ºC (2 min). Then we found the CT (cycle at the threshold) value determined for the target gene where fluorescence data crossed the present threshold. The threshold is calculated as 20 times the standard deviation (SD) of the average signal of fluorescence values for the baseline representing the normalized value established in the first few PCR cycles. All samples reaching the threshold were considered to be positives [23-24].

The B1 primer was also tested with conventional PCR method. The B22 and B23 primers were used for amplifying a 115 bp sequence from the B1 gene in order to *T. gondii* DNA detection according method described by Bretagne et al. [25]. Each 25 µL PCR solution contained 0.5 µL DNA, 50 pM primers, 0.2 mM deoxynucleoside triphophate (Fermentas Inc., Hanover, MD, US), 1.5 mM Mgcl2 and 2.5 U of Taq DNA polymerase (Fermentas Inc., Hanover, MD, US).

PCR amplification protocol was as follows: denaturation at 94 ºC for 4 min; 35 cycles of 94 ºC for 45 s, 58 ºC for 45 s and 72 ºC for 1 min and an extension of 72 ºC for 10 min. A 2% agarose gel was then used to visualize of amplified PCR products by electrophoresis [26]. After the experiment above mentioned was finalized, the acquired data was assessed by statistical methods (t-test and chi-square test) in SPSS software.

RESULTS

According to Table 1, a total of 65 abortion cases were identified during the study period (mean: 27.43±6.24 yr, range: 17-75 yr). Of these women, 12 (18.5%) had a previous history for abortion (mean: 8.95±3.96 weeks).

According to Table 2, of the 55 patients without appendicitis, 2 (3.1%) reported a history of hypothyroidism, 1 (1.5%) with

Table 1. The characteristics of Iranian abortion cases

Characteristics	
Age (yr)	**27.43±6.24**
Range (yr)	17-45
Abortion Gestation (wk)	**8.95±3.96**
Range (wk)	0-19
Previous abortion	
Yes	**12 (18.5%)**
Once	10 (15.4%)
Twice	1 (1.5%)
thrice	1 (1.5%)
No	**53 (81.5%)**

Table 2. The disease history of Iranian abortion cases

Disease history	**No (%)**
Yes†*	**18 (27.7)**
appendices	10 (15.4)
hypothyroidism	2 (3.1)
thalassemia	1 (1.5)
diabetes	2 (3.1)
Allergy	3 (4.6)
No	**47 (72.3)**

† chi-square, * P≤0.05

Table 3. Conventional PCR results for toxoplasmosis in Iranian abortion cases

	Toxoplasmosis	
	positive	negative
Conventional PCR*	10 (15.48%)†	55 (84.6%)

† chi-square, * P<0.05

Table 4. Real time PCR results for positive toxoplasmosis in Iranian abortion cases with or without appendectomy

Variables	Appendectomy	
	yes	no
Real-time PCR*	13.18±5.86†	0.38±0.06
No (%)	8 (80%)	2 (20%)

† t-test, * P<0.05

thalassemia, 2 (3.1%) with diabetes, 3 (4.6%) with allergy. Fetal dissemination occurred within 2-18 weeks of gestation (mean: 9 weeks) was the only history in the remained 47 patients. The results of chi-square test showed that there was a difference among those who had a history and those had not (P≤0.05). Eight (84.6%) had appendectomy while 2 (15.48%) did not undergo a surgery on appendicitis. Also, Out of 12 cases with previous abortion, 10 (15.4%), 1 (1/5%) and 1 (1.5%) had an abortion for once, twice and thrice times, respectively in the past.

As Table 3 shown, positive findings were obtained for 10 of 65 (15.48%) analyzed tissue sections and a significant difference was observed between two groups of positive and negative *T. gondii* (P≤0.05). The real-time PCR CT values ranged from 30.70 to 39.2. These results are concordant with those of some conventional PCR methods (P<0.05). Eight (80%) of 10 women with appendices had to undergo an appendectomy and the average number of obtained parasites is 10.63 (Table 4) which were significantly higher in surgical patients in this assay (P≤0.05); that is appendectomy was associated with infection in immunosuppressive cases.

DISCUSSION

Until now, studies concerning *T. gondii* have never been conducted in appendectomy cases with the usefulness of the two techniques, i.e. conventional and real time PCR. When assessed in our patients, 10 from 65 patients were positive for *T. gondii* with both conventional and real-time PCR. In most instances, toxoplasmosis develops when CTs falls below 40 [24]. The CTs range of 30.7 to 39.2 in our survey is in accordance with such data. The only association that we found was for rate of infection in abortion in cases undergone appendectomy detected by real time PCR, while the two other women had a statistically significant lower quantity of agent in their real time PCR assay.

T. gondii in pregnant women showed the following statistics among some epidemiologic populations screened by variant diagnostic methods: In Palestine, 7.9% in 2003 (N=1954, P=0.001) [27]; in Spain, 44% in immigrants vs. 14.4% in non-immigrants (N=4171, 2007 to 2008) [28]; in Morocco, 50.6% by ELIZA (N=2456) [29]; In Colombia, the estimated 30-120 per 8000 pregnancy (N=937) [30]; in Latin America, 51-72% [31]; in Denmark, 33% estimated in 1990 [14], also 27% by enzyme immunosorbent assay for IgG and IgM in 1995 (N=5402) [32]; In Mexico, 47% by Sabin-Feldman test in 1989 (N=100) [33]; In Paris, 71% in French women and 51.4% in immigrant women and a global estimate of 67.3% between 1981 to 1983 (N=1074) [34]. Also, latent infections were detected in some study design [35].

We obtained the prevalence of 15.48% in Iranian women with recent abortion. Our value is nearly consistent with results of the study in Spain in which 14.4% of Hispanic pregnant women were seropositive for toxoplasmosis. In Kashmir, it is estimated that 49.47% of women with repeated abortions (N=285) were detected to be positive for IgM toxoplasma antibody [36]. In another study in Mexico, women with a history of abortion had a significantly higher seroprevalence of *T. gondii* IgG as researchers found *T. gondii* seroprevalences of 44.9% in women with habitual abortions from central Mexico and 47% in women with spontaneous abortions from the south of Mexico [37].

On the other hand, the patients who have a weak immune system are more vulnerable to *Toxoplasma gondii* [24]. The true statistics among immunocompromised patients is difficult to assess for overlooked diagnosis in many cases [38]. However, a revolutionary improvement in diagnosis has occurred based on the technological ability of the polymerase chain reaction (PCR) to detect and identify any infectious agent especially latent infections rapidly and efficiently [39]. Also, PCR has been shown to be sensitive in immunocompromised patients affected by toxoplasmosis and may function as a better option for early identification of infection [38]. After the first application of PCR for *T. gondii* in ocular specimen performed in 1990 [14], Aouizerate et al. found an infection rate of 33.8% in 59 cases with parasitic eye infections by PCR [15]. Although the sensitivity for detection of foreign DNA is very high, the high sensitivity and specificity can cause significant pitfalls. Therefore, utilizing a secondary detection system in concert with the initial PCR reaction leads to perfect specificity [15]. Villard et al. (2003) also reported the high yield of a combination of serologic analysis enzyme-linked immunosorbent assay (ELISA), immuo-blotting and PCR for ocular toxoplasmosis in 83% (15 of 18) of patients [19]. The relative specificities of the three techniques were 89% for ELISA and immuno-blotting but 100% for PCR. Also in another study, the probability of diagnosing ocular toxoplasmosis increased from 60% to 72.7% by the combination of PCR with serologic analysis [18].

However, the major disadvantage of the conventional PCR-based techniques is their time-consuming procedure without ability for providing quantitative data. This was removed by the recent advent of a real time quantitative PCR technique. This methodology facilitates *T. gondii* diagnosis in various fields like pathogen detection, gene expression and regulation, and allelic discrimination. In comparison with the conventional PCR, major advantages of real-time PCR are its ability to quantify the infection discrimination [24]. Quantification of infection load has been used to assess disease severity and treatment outcome in human immunodeficiency virus and hepatitis C virus infections and also has application in comparing different drug regimens and in determining the prognostic value of treatment [24,40]. Lin, et al (2000) in 2000 developed a real-time PCR-

based B1 gene-specific TaqMan assay for quantitative detection of *T. gondii* [24]. They have demonstrated that real-time PCR of the B1 gene is extremely sensitive (0.05 parasite/reaction) and highly reproducible (mean inter-assay CV of 0.4%). Then, Alfonso et al (2009) concluded that PCR using the B1 gene and B22/B23 set of primers is a single, rapid and reliable method that may be valuable for diagnosis toxoplasmosis [41].

Thus, this method may be suitable particularly for screening AIDS patients without generation of specific IgM or increased IgG titers. As an important opportunistic agent, *T. gondii* affects organ transplant recipients, AIDS patients, and other immunocompromised patients [24]. Some statistics for *T. gondii* are addressed in relation to immunosuppression. In France, CNS toxoplasmosis was detected in 13.8% of AIDS patients in 1994 [42]. The cerebral toxoplasmosis is the second most common opportunistic infection (OI) in HIV-infected individuals in developing countries that causes of morbidity [12]. In a group of 33 clinically suspected cases of AIDS-associated cerebral toxoplasmosis from North India, 72% were reported to have high serum IgG titers [43]. Our findings presented higher *T. gondii* DNA load in appendectomy cases, as detected by real time PCR. Only 20% of our positive cases did not undergo appendectomy and a positive relationship was found for the load of agent between women with appendectomy and women without. This finding is consistent with those of other studies in which all patients with TE had a positive PCR, indicating that *T. gondii* was already present in the case of immunosuppression. The conventional PCR shows a less impressive sensitivity for such cases as Dupon et al. [42] found a range from 35.3 to 100% for HIV-infected patients.

In our study, the results of real time PCR revealed that in the patients who had done appendectomy, number of *T.gondii* infections was higher than others. Therefore, as a result of appendectomy one is encountered with immunodeficiency. So, it is for the first time in Iran that we could find a relationship between appendicitis and lack of a strong immune system and the results of real-time PCR revealed that in the patients who had done appendectomy, number of *T. gondii* was more than others. So, it is recommended next to study about *T. gondii* and appendectomy separate from abortion.

We cannot rule out that our results were confounded because real time PCR is a quantitative PCR with a high specificity. Because our study gave a contribution to the screening of pregnant women done in Isfahan, Iran during logic time period, the results can be representative for epidemiology of Iranian pregnant women. However, toxoplasma infection in the study area can lead to some interpretations for the pattern of risk factors, surveillance and prevention programs [2,6]. Also, the found association for appendectomy and abortion is primarily of medical importance for immunocompromised patients who are at risk of affecting by the severe disease that may be fatal [1,41].

Use of a study population of pregnant women affects generalizeability of findings; nevertheless, results indicate that toxoplasmosis is a major threat for health of pregnant women and their fetus in this area of Iran. This problem highlights the need for improved education programs, especially for population with immunocompromised status. The Real-time PCR is a strong tool applied here for epidemiologic analysis of self-reported data. It can be used for improving effectiveness of invention programs.

Conclusion:

In conclusion, toxoplasma infection in the study area was high in pregnant women. Our finding that 80% of infections were associated with appendectomy indicates that immunosuppressive conditions were major source of infection induction in the study population. It is probably to vary by population; therefore future studies should show if the values are caused by chance or unknown confounders, or mathematical models should be applied based on the prevalence of T. gondii DNA as a useful estimation of the magnitude of the problem.

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