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Original Article

Molecular Genotyping of *Toxoplasma gondii* in Human Spontaneous Aborted Fetuses in Shiraz, Southern Iran

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Abstract

Background: Congenital toxoplasmosis is associated with variable complications including encephalitis, microcephaly, hydrocephaly, hepatitis, lymphadenopathy and even intrauterine death. Presence of *Toxoplasma gondii* in human placenta may induce congenital infection. The aim of this study was to determine the genotypes of *Toxoplasma gondii* infection in human spontaneous aborted fetuses in Shiraz, south of Iran.

Methods: Five hundred and forty two paraffin-embedded blocks of aborted placenta were collected, from two university-affiliated hospitals in Shiraz. Occurrence of spontaneous abortion was confirmed by examine of the slides. After re-cutting of the blocks and dewaxing, semi-nested PCR assay was used to detect the fragments of *T. gondii* B_1 gene in the samples. Also direct molecular genotyping was performed on positive samples with Restriction Fragment Length Polymorphism-PCR analysis on the SAG2 gene.

Results: Among the 542 tissue samples, the B1 gene was amplified from 78 (14.4%) of cases with the semi nested PCR and typed by RFLP. The genotype of *Toxoplasma* strains of 65 (out of 78) PCR-positive samples were evaluated and 54 out of 65 (83.1%) were found to be type II and 11 out of 65 (16.9%) were type I.

Conclusion: Considering the high level of *Toxoplasma* infection in aborted fetuses in this study, *Toxoplasma* might largely contribute to spontaneous abortion in this area of Iran.

Keywords: *Toxoplasma gondii*, Abortion, Genotype, Iran

Introduction

Toxoplasma gondii is one of the most common protozoa that infect humans and a wide range of mammalians and birds (1). Most cases of acquired toxoplasmosis are asymptomatic or may cause flu-like disease. However, in immunocompromised patients, such as HIV infected and organ transplant recipients, *Toxoplasma gondii* may cause a severe life threatening disease, resulting in brain lesions or diffuse encephalitis. Reactivation of latent infection has been accounted for the cause of severe infection in such individuals although severe toxoplasmosis can also result from acute infection (2).

Acquired infection in a previously immunologically naive pregnant poses a risk of congenital infection whereas previously infected mothers due to development of strong immunity rarely transmit the disease during following pregnancies (2). Maternal toxoplasmosis during early pregnancy may lead to death of fetus, stillbirth, mental

retardation, hydrocephaly, microcephaly, seizures and retinal damage. Moreover, recurrent toxoplasmic chorioretinitis in children and young adults is mainly the result of congenital infection in individuals who are asymptomatic at birth (2).
It has been estimated that congenital toxoplasmosis affects 1 to 10 per 10,000 infants in Europe (3). Its incidence and severity varies depending on the time of infection in mothers. Rate of transmission may increase with gestational age whereas the severity of infection is reduced by the time of infection (4-5).
Moreover, the role of parasite virulence must be considered in severity of infection. Despite the existence of sexual phase in the life cycle of the parasite, the population genetic structure of *T. gondii* is extremely clonal, and molecular genotyping has shown that approximately 90% of the *T. gondii* isolates from different hosts can be classified into three clonal lineages; types I, II and III (6).
Toxoplasma gondii is contributed to the abortion and the rate of abortion due to congenital toxoplasmosis is unclear in many areas where the rate of acquired toxoplasmosis is reasonably high.
The current study aimed to find out the rate of *Toxoplasma* infection in human spontaneous aborted fetuses of human in Shiraz district, south of Iran. The study was justified by the lack of such information in this area.

Materials and Methods

Samples of spontaneous aborted fetuses

Five hundred and forty two paraffin-embedded blocks of aborted placenta, which were collected during 2005 to 2009, from two university-affiliated hospitals in Shiraz, were used in this study. All the samples were evaluated once more and occurrence of spontaneous abortion was confirmed in all samples.

DNA extraction from paraffin-embedded blocks

The paraffin-embedded samples were deparaffinized as described by Wright and Manos (7). Briefly, 5-µm sections of paraffin-embedded placenta were cut and transferred to a 1.5 ml microcentrifuge tube and deparaffinized by xylene extraction. One milliliter of xylene was added to each tube and mixed, at room temperature, for an hour. Tissue and residual paraffin were then pelleted by centrifugation at 14,000 rpm for 5 minutes. After a second xylene extraction, the xylene was removed by pipetting. The samples were then washed twice with 100% ethanol to remove the organic solvent. The sediment of last washing step was homogenized and diluted with proteinase K (200 µg/ml) and lysis buffer (0.5 ml of Tris–HCl, pH=7.6; 1 mM of EDTA, pH=8.0; 1% Tween 20) and incubated for 24 hour at 37°C. The lysate was then extracted twice with phenol/chloroform/ isoamyl before the DNA was precipitated with absolute ethanol. The precipitated DNA was resuspended in 100 µL of double-distilled water and stored at 4°C until use.

Amplification of DNA by Semi-Nested Polymerase Chain Reaction

A semi-nested PCR was used to amplify the segments of the 35-fold repetitive DNA region of B1 gene of *T. gondii* as essentially described by Hill et al., (8). The forward; 5'-GAACTGCATCCGTTCATG-AG -3' and reverse; 5'-TCTTTAAAGCGTTCGTGGTC-3' primers were used to amplify the related target. Cycling conditions for both the direct and semi-nested PCR were denaturation at 94 ºC for 5 min, followed by 40 cycles of 94 ºC for 1 min, 60 ºC for 1 min, and 72 ºC for 2 min. A 5- µl sample of each PCR product was subjected to electrophoresis in 1.5% agarose gel.
The bands were then stained with ethidium bromide and visualized under ultra-violet trans-illumination. Positive sample (*Toxoplasma gondii* RH strain) (kindly provided by Dr Keshavarz, from Tehran University of Medical Sciences) was used in this study.

Genotyping of Toxoplasma

Molecular genotyping was performed on PCR positive samples, using Restriction Fragment Length Polymorphism-PCR (RFLP) analysis on the SAG-2 gene.

Amplification of the 3' end was performed with SAG2F3 (5'-TCTGGTTCTCCGAAGTGACTCC-3') and SAG2R3 (5'-TCAAAGCGTGCATTATCGC-3') primers for the first round of amplification and with the internal SAG2F2 (5'-ATTCTCATGCCTC CGCTTC-3') and SAG2R (5'-AACGTTTCACGAA GGCACAC-3') primers for the second round of amplification. The PCR reactions were performed in a programmable thermocycler (Corbett Research, Australia). The first 25 µL of PCR reaction mixture contained outer primers at a final concentration of 50 pmol each, 2.5 mmol dNTPs, 1 µg of template, and 1.5 U of recombinant *Taq* DNA polymerase (GENET BIO, Korea, A-type Prime *Taq*TM DNA polymerase), in PCR reaction buffer (50 mmol/L KCl and 10 mmol/L Tris–HCl, 1.5 mmol/L $MgCl_2$, and 0.1% triton X-100; CinnaGen Co., Iran). The first step of amplification was 5 min of denaturation at 94°C. This step was followed by 40 cycles, with one cycle of 45 s at 94°C, 45 s at the annealing temperature 58°C for first amplification but 55°C for second amplification, and 60 s at 72°C. The final cycle was followed by an extension step of 10 min at 72°C. The PCR products were digested, using 5 U of *HhaI* (3'-end products). Restriction fragments were separated on 1.5% agarose gel in TBE (Tris Base/Boric Acid/EDTA) buffer.

Preparation of stained slides

The paraffin-embedded tissues, which were positive by PCR were sectioned at 5 µm thickness and stained with Giemsa and hematoxylin-Eosin stains.

Analysis of data

The results were analyzed by SPSS software (version 13), using Chi-square test and a *P* value less than 0.05 was considered as statically significant.

Results

Using PCR method, 78 out of 542 (14.4%) samples of spontaneous aborted fetuses were found to be positive for *T. gondii* (Fig. 1).

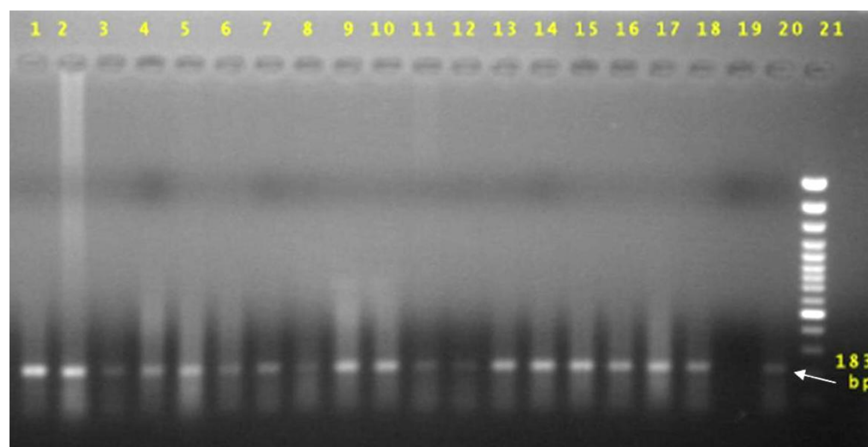

Fig. 1: The amplicons produced, in the semi Nested PCR based on the B1 gene primers, from aborted placenta. The amplified product of 183 bp belonged to *Toxoplasma*. /Lane 1-18: positive tissue of placenta; lane 19: negative sample, lane 20: positive sample (*Toxoplasma gondii* RH strain), Lane 21: DNA ladder

Products of the second round of semi nested PCR was used for RFLP analysis. The 183 bp-amplified fragment was digested with HhaI and the results showed the pattern of type II and III (Fig. 2). According to our findings, 54 out of 65 (83.1%) of samples were type II and 11 out of 65 (16.9%) were type III of *T. gondii*. The PCR-positive slides were stained with Giemsa and hematoxylin & Eosin to find out any forms of the parasite, mainly tissue cyst or tachyzoites, in the samples. None of the samples was positive for tachyzoites or tissue cysts of the parasite.

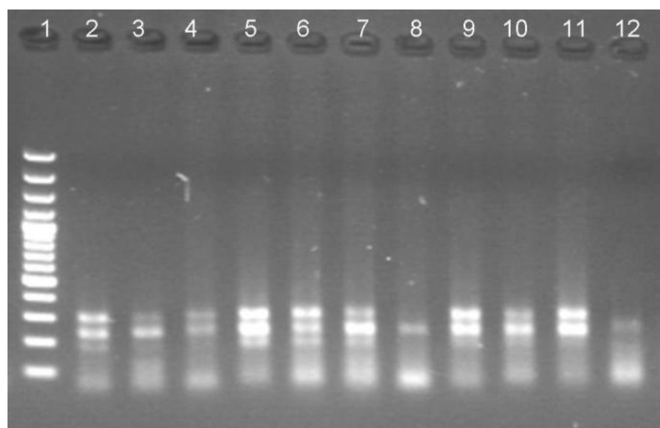

Fig. 2: Restriction enzyme analysis of PCR products from *Toxoplasma* positive aborted tissues digested with *HhaI*. Lane 1: 100 bp DNA ladder; Lane 1-7 and 9-11: type II of *Toxoplasma*, lane 8: type 1, lane 12: positive control (type 1; RH strain)

Discussion

Toxoplasmosis is a worldwide infection, which contributes to spontaneous abortion in human and animals. Its seroprevalence is as high as 50 percent in some areas of the world including some regions of our country (9).

During pregnancy, *Toxoplasma* infection might be a serious risk factor for fetus due to congenital toxoplasmosis. Rate of infection in pregnant women in Iran is relatively high. This rate in Zahedan, southeast of Iran, was reported to be 27% while in north of the country was 43.8% (10-11). In Shiraz, Southern Iran, the seroprevalence of toxoplasmosis among pregnant women was reported to be 77.2% (12). This rate is lower in high school girl in the region (13).

In a study on pregnant women in New York, 0.6% of mothers acquired *Toxoplasma* during pregnancy and 13% of their infants born with congenital toxoplasmosis. So the rate of congenital toxoplasmosis was reported to be 7 per 10,000 live births (14).

A study in Colombia showed that 61 out of 15,333 umbilical cord blood samples have specific IgM for anti-*Toxoplasma*. This contributed a rate of 39 per 10,000 live births for congenital toxoplasmosis in this area (15). A study on seroprevalence of toxoplasmosis in Kosovo in pregnant women demonstrated that 1.2% of women acquired toxoplasmosis during their pregnancy (16).

In our study, the rate of *Toxoplasma* infection in aborted placenta was 14.4%. This is higher than the rates that have been reported for abortion due to toxoplasmosis in other studies in the world. One explanation for this high prevalence of *Toxoplasma* in aborted samples in our study might be the using of molecular method, PCR, for detection of the parasite. Type of sample, method of sampling, and size of sample may all affect the outcome of the experiment. Moreover, detection of *Toxoplasma* DNA in placental tissue might not exclusively be connected to the abortion.

Most of *T. gondii* isolates from human and animal sources clustered into three clonal types, I, II and III (6, 17). Differences in virulence in laboratory animals are one of the most determined variability between these lineages. Type I is responsible for the lethal infection in out bred mice while types II and III are less virulent. In North America and Europe, type II strains are most commonly associated with human toxoplasmosis (17).

In our study 54 out of 65 (83.1%) of samples were type II and 11 out of 65 (16.9%) were type III of *T. gondii*. Using PCR-RFLP analysis, the genotypes of 18 *T. gondii* strains isolated from humans in Crete and Cyprus were belonged to type II (22%) and type III (78%) (18).

The possible association between ocular or congenital toxoplasmosis and *T. gondii* genotype has been evaluated in several studies. Genotyping of *T. gondii* strains isolated from blood samples of patients with ocular toxoplasmosis showed that all detected strains belonged to type I, suggesting an association between ocular toxoplasmosis with the type I of *Toxoplasma* (19). However, such connection has not been reported in other studies (20).

Genotyping of the *Toxoplasma* isolated from infants with congenital toxoplasmosis in Poland demonstrated type II, as the dominant type of *T. gondii* infection in congenital toxoplasmosis (1).

Abdel-Hameed (2008) evaluated the genotype of *Toxoplasma* in thirty-eight female patients with abortion and intrauterine fetal death in Egypt, using PCR-RFLP assay at SAG2 locus. Of the 38

isolates, type II was the most prevalent genotype found in 33 (87%) and type I was found in 5 (13%) of the isolates (21).

Taken together, considering the high level of *Toxoplasma* infection in aborted fetuses in this study, *Toxoplasma* might largely contribute to spontaneous abortion in this area of Iran. This highlights the importance of the infection during pregnancy and, in turn, the significance of control measurements during pregnancy.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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