

## GENETIC POLYMORPHISM OF *TOXOPLASMA GONDII* FROM RECENTLY INFECTED ABORTED EGYPTIAN WOMEN

By

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### Abstract

Toxoplasmosis caused by *Toxoplasma gondii* is one of the most prevalent parasitic diseases in human beings. Human toxoplasmosis can be associated with serious clinical manifestations, particularly in developing fetus. The aim of the current study was to identify the possible lineage type of *Toxoplasma gondii*, molecularly detected in placental samples of women whose pregnancies were spontaneously terminated in the first trimester. Preliminary detection of *Toxoplasma* genomic materials was done by a SYBR green qPCR technology. Subsequent identification of *Toxoplasma* strain was done for the positive samples using PCR- restriction fragment length polymorphism (RFLP) at the *SAG2* loci of *T. gondii* using restriction enzymes *HhaI* and *Sau3AI*. Out of 72 tested samples, *Toxoplasma* B1 gene was detected in 9 cases. *Toxoplasma* genotypes I and II in addition to unknown type were identified in 4, 3 and 2 cases respectively, while type III was not detected in our samples, hence excluded as a leading cause of abortion in humans in our preliminary study. Nevertheless, it remains uncertain to what extent the genotype of the parasite directly contributes to the clinical severity of human toxoplasmosis. Certainly, advanced molecular techniques targeting different *Toxoplasma* strains are crucial for better understanding of human toxoplasmosis. For more elucidation, additional studies are recommended intended for genetic characterization of such serious parasitic infection using larger number of samples.

**Keywords:** abortion, genotype, RFLP, *Toxoplasma gondii*

### Introduction

*Toxoplasma* infection is endemic worldwide and asymptomatic human infections varied from 15 to 85% according to the geographic location (Sarker *et al*, 2012). Primary *Toxoplasma gondii* infection during pregnancy can be transmitted via placenta to the fetus in about 30% of cases (Desmonts and Couvreur, 1984). Infection acquired during pregnancy can cause stillbirths or fetal death (Montoya and Liesenfeld, 2004). It is documented that toxoplasmosis is one of the causative agents for abortion and congenital abnormalities outcome (Nazar *et al*, 2015). The possibility and severity of congenital toxoplasmosis basically depend on the gestational age at which the mother acquired the infection. It has been established that the congenital infection is severe, ranging from intra uterine

fetal death or abortion to severe neurophthalmic involvement when the infection is acquired during early phase of pregnancy (Dunn *et al*, 1999; Ajzenberg *et al*, 2002). It is reported that the parasite load could play a role in pathogenicity and severity of the infection (Gross *et al*, 1997). Quantitative polymerase chain reaction (PCR) suggested an association between high parasite count in amniotic fluid and ultrasonographic abnormalities, although the main factor remains early maternal infection (Costa *et al*, 2001).

The question of relationship between congenital toxoplasmosis and *T. gondii* genotype has been approached in several studies, with occasionally discordant results. For biologic and epidemiologic studies, 3 main clonal lineages are generally recognized in the *T. gondii* population, types I, II

and III (Howe *et al.*, 1997). These 3 major clonal lineages based on genotypes are type I (includes strains like RH which are highly virulent), type II (includes avirulent strains like PLK and Beverley), and type III (includes avirulent strains like VEG and CTG) (Howe and Sibley, 1995). These classical genotypes are previously identified applying restriction fragment length polymorphism (RFLP) which is commonly used for *T. gondii* strain identification in PCR-amplified *SAG2* loci (Khan *et al.*, 2006). The possibility of performing the technique directly on clinical samples including blood has been previously documented by Fuentes *et al.* (2001).

The consequences of infection with *T. gondii* are also related to parasite genotypes and the genetic make-up of the parasite plays an important role in the course of toxoplasmosis (Suzuki *et al.*, 1989; Jungerson *et al.*, 1999; Johnson *et al.*, 2002). The type I strains are associated with severe or atypical ocular toxoplasmosis in infected immunocompetent adults and are over-represented in congenital infections (Grigg and Boothroyd, 2001). On the contrary, type II strains account for most clinical toxoplasmosis cases in immunocompromised patients (Howe and Sibley, 1995; Grigg and Boothroyd, 2001). While, type III strains are found in patients with ocular toxoplasmosis (Fux *et al.*, 2003). Exposing the possible correlation between the severity of the disease and strain genotyping might be of value to identify the outcomes of the infection and hence proper management of infected cases.

In general, the distribution of *T. gondii* genotypes varies in geographic regions (Lehmann *et al.*, 2006). Thus, the aim of the present study is to determine the genotypes of *T. gondii* molecularly detected in placental samples and associated with abortion in primarily infected Egyptian women using restriction fragment-length polymorphism (RFLP) in PCR-amplified *SAG2* loci.

## Patients, Materials and Methods

Considering the ethical issues, cases included in this study were selected from the outpatient's clinic of Obstetrics and Gynecology Department in Kasr Al Ainy Hospital, during the period from January 2013 to September 2015. A total of 72 women, whose pregnancies were spontaneously terminated at less than 14 weeks were included in the study. Blood samples were collected from all cases and sera were separated and were tested for anti-*Toxoplasma* IgG antibodies using a commercial ELISA Kit (Cal biotech Inc., CA). The assay was carried out according to the manufacturer's instructions. Immune women proved to be seropositive for anti-*Toxoplasma* IgG were intentionally excluded from the study; therefore the study was conducted on the 72 non immune women. To confirm primary recent *Toxoplasma* infection, Preliminary detection of *Toxoplasma* B1 gene in the placenta tissue was done by qPCR applying SYBR Green technology. Subsequent identification of possible *Toxoplasma* strain was done for positive samples using PCR-RFLP at the *SAG2* loci of *T. gondii*.

DNA extraction and amplification by Quantitative real time polymerase chain reaction (PCR): Genomic DNA from placental biopsies was extracted using the DNeasy<sup>®</sup> Blood and Tissue Kit (Qiagen, CA, USA). Quantitative real time PCR (qPCR) was performed according to Contini *et al.* (2005) with the LightCycler<sup>®</sup> fastStart DNA Master SYBR Green dye, using the LightCycler<sup>®</sup> instrument (Roche Diagnostics, Hoffmann-La Roche Ltd, USA). Primers from bases (5'-CCG TTG GTT CCG CCT CCT TC-3') and (5'-GCA AAA CAG CGG CAG CGT CT-3') were used to amplify *Toxoplasma* B1 gene of 35-fold repeats. The resulting PCR fragment of *T. gondii* was analyzed using the Light Cycler<sup>®</sup> Red 640 (detected in channel 640). The reaction mixture (20  $\mu$ l; Master SYBR Green kit; Roche Diagnostic) contained 0.5  $\mu$ M of each primer, 5 mM

MgCl<sub>2</sub> and 5 µl template DNA. Amplification was performed for 50 cycles: 5 s denaturation at 95°C, 10 s annealing at 61°C and 15 s extensions at 72°C, with an overall ramp rate of 20°C/s. Positive sample specificity was confirmed by determining the melting curve with different values of melting temperatures (*T<sub>m</sub>*) (95°C, 4.40°C/s ramp rate; 40°C, 2.20°C/s ramp rate; 65°C, 4.40°C/s ramp rate, 95°C, 0.02°C/s ramp rate continuous measurement. A standard row was generated using the provided cloned and purified *Toxoplasma* DNA (Roche Diagnostics), to allow for the absolute quantification of the unknown samples. Negative control was applied by replacing the template DNA with water.

Restriction Fragment-Length Polymorphism (RFLP): Positive samples by qPCR were subsequently analyzed by RFLP for *SAG2* gene using restriction enzymes *HhaI* and *Sau3AI*. Genotype analysis was performed according to Fuentes *et al.* (2001), the lineage type was determined by restriction fragment of the amplified *SAG2* gene of *T. gondii* using two nested PCRs separately amplifying the 5' and 3' ends of the gene. All primers and restriction enzymes used were provided by Pioneer Company, USA. The 5' end was amplified using primers *SAG2F4* (5'-GACCTCGAACAGGAACAC-3') and *SAG2R4* (5'-GCA TCAACAGTCTTCGTTGC-3') in the first amplification, at an annealing temperature of 60°C. In the second reaction, the internal primers *SAG2F* (5'-GAAATGTTTCAGG TTGCTGC-3') and *SAG2R2* (5'-GCAAGA GCGAACTTGAACAC-3') were used with 58°C as the annealing temperature. The final product was digested with *Sau3AI*. Amplification of the 3' end was performed with primers *SAG2F3* (5'-TCTGTTCTCC GAAGTGA CTCC-3') and *SAG2R3* (5'-TC AAAGCGTGCATTATCGC-3') for first

amplification at an annealing temperature of 58°C and with internal primers *SAG2F2* (5'-ATTCTCATGCCTCCGCTTC-3') and *SAG2R* (5'-AACGTTTCACGAAGGCAC AC-3') for the second amplification at an annealing temperature of 55°C. The final product was digested with *HhaI* and all products of digestion were analyzed by 3% agarose gel electrophoresis. Restriction digestion of 5'-end-amplified products with *Sau3AI* distinguished type III strain (which is digested by *Sau3AI*) from type I and II strains (being not digested) and digestion of the 3'-end-amplified fragments with *HhaI* differentiated type I and II strains (strain II not digested by the enzyme).

### Results

Applying qPCR in this study on the 72 tested samples, *T. gondii* B1 gene was detected in 9 samples (12.5%). Genetic analysis was then performed for these positive samples by PCR-RFLP at the *SAG2* loci of *T. gondii*. Separate amplification of *SAG2* gene (5' end) by nested PCR showed products at 241 bp, and of *SAG2* gene (3' end) at 221bp. For identification, genotype I was not affected by both *Sau3AI*enzyme and *HhaI* restriction enzyme, while genotype II digested with *HhaI* restriction enzyme & did not digest with *Sau3AI* enzyme. Genotype III digested with *Sau3AI* enzyme and not digested with *HhaI* restriction enzyme (table 1 and fig. 1). In our cases, successful amplification of both ends of the *SAG2* gene was observed in 7 out of these 9 cases (77.8%), while the remaining 2 cases, only one end of *SAG2* gene was amplified, so, these 2 cases were classified to be of unknown genotype. The genotypes of positive cases were 4 cases (44.4%) of type I, 3 cases (33.3%) of type II and 2 cases (22.3%) of unknown genotypes. Genotype III was not detected in any of the cases.

Table 1: Number and percentage of cases related to different genotypes

Genotype I		Genotype II		Genotype III		Unknown Genotype		Total
No.	%	No.	%	No.	%	No.	%	
4	44.4%	3	33.3%	0	0%	2	22.3%	9 (100%)

## Discussion

*Toxoplasma gondii* is an obligate, intracellular protozoan parasite distributed worldwide. Infection in humans is usually asymptomatic, but it can manifest itself in a severe form in cases of congenital toxoplasmosis and in immune-compromised individuals (Dubey *et al.*, 2012a). Congenital *Toxoplasma* infection can lead to severe neurological problems or even death (Wong and Remington, 1994). Rapid and accurate diagnosis is required to start antiparasitic treatment. The traditional diagnosis of *T. gondii* infection usually depends on bioassays and serological tests, with the limitations in detection or differentiating parasite strains (Bastien, 2002; Kotresha and Noordin, 2010). Detection of the parasite DNA by PCR has considerably improved prenatal diagnosis of congenital disease (Bastien, 2002). The real-time PCR assay with the B1 gene is considered as the best-performing technique for diagnosis of congenital toxoplasmosis, compared with conventional PCR and nested-PCR (Teixeira *et al.*, 2013). In the present study, a total of 72 placental biopsies samples, obtained from non-immune women who developed abortion, were tested for *Toxoplasma* genomic material by qPCR. Consequently, *Toxoplasma* B1 gene was detected in 9 out of these 72 cases.

*T. gondii* isolates obtained from humans have been classified into one of three genetic clonal lineages, Types I, II, and III (Howe and Sibley, 1995). In general, genotyping studies are important for understanding the population structure and parasite phylogeny and may help identify the sources of infection or means of transmission to humans (Su *et al.*, 2010). PCR-RFLP was applied in the present study to determine the clonal lineage of *Toxoplasma* in positive samples for *Toxoplasma* genomic material. The PCR-RFLP is based on the ability of restriction endonucleases to digest PCR products and subsequently

display distinct DNA banding patterns on agarose gels electrophoresis (Howe and Sibley, 1995).

In the present study, genotypes I & II in addition to unknown genotype were detected in placental biopsies obtained from women whose pregnancies were spontaneously terminated. In humans, the dichotomy between virulent and avirulent strains of *T. gondii* is not as clearly reflected at the clinical level as in mice (Boothroyd and Grigg, 2002), and attempts to establish a correlation between the genotype of *T. gondii* and a given disease manifestation has yielded conflicting results. Some studies on humans suggested that type I strains are highly virulent and the most prevalent cause of infection in immunocompromised patients, whereas type II and type III are relatively nonvirulent (Sibley and Boothroyd, 1992). A CS3 marker, located on *T. gondii* chromosome VIIa, was shown to be linked to the acute virulence of clonal type I *T. gondii* strains (Khan *et al.*, 2005). Moreover, a study of Brazilian *T. gondii* isolates showed that the alleles type I and II at the CS3 locus are strongly linked to mouse virulence of the parasite (Pena *et al.*, 2008).

However, in most studies in North America and Europe, type II strain was recorded to be the most prevalent cause of human toxoplasmosis, both in congenital infection and in AIDS patients. Other studies reported that type II genotype is the most prevalent one, with values between 70 and 81% and recorded its association with all kinds of human toxoplasmosis (Fuentes *et al.*, 2001). Furthermore, all congenital toxoplasmosis cases studied by Howe *et al.* (1997) were reported to be of type II. On the other hand, Fuentes *et al.* (2001) found that only 8% of congenitally infected cases were type II, 46% were type I, 8% were type III and 38% were non-type II. Moreover, they found a maximum of 55% of infection by *T. gondii* type II in the immuno-

compromised patients. They suggested that the epidemiological prevalence and the route of transmission may be very different in different regions, where studies were performed. The use of cell culture and grow the parasites from the clinical samples, as has been the case in many previous reports, might produce sensitive variations in the observed genotyping frequencies due to an effect of differential selection of the strains (Fuentes *et al.*, 2001). In France, where a systematic diagnosis of congenital toxoplasmosis was performed, more than 80% of infections were caused by the archetypal genotype II (Ajzenberg *et al.*, 2002).

In humans, genotype II strains are responsible for 70% of all symptomatic infections, whereas genotype III isolates are relatively rare (Howe and Sibley, 1995). In accordance with that, genotype III was not detected in our cases. On the other hand, Dubey *et al.* (2003) and Dubey *et al.* (2005) documented the first reports about *T. gondii* genotypes in Egypt and several other African countries found a predominance of type III genotypes based on the analysis on single *SAG2* marker, taking in to account that the authors applied the technique on avian samples, not human specimens.

Among the various markers used for *Toxoplasma* genotyping, the *SAG2* marker provides accurate genotyping for most strains within the clonal lineages (Fazaeli *et al.*, 2000). Therefore the technique was applied in our study on human samples rather than animal specimens. This issue is important as strain-specific virulence varied between hosts and it was suggested that *T. gondii* strains virulent for mice might not lead to a similar course of toxoplasmosis in humans (Zenner *et al.*, 1999; Dubey *et al.*, 2012b).

### Conclusion

The molecular techniques studying different *Toxoplasma* strains are important for better understanding of epidemiology and

association between parasite genotypes and human toxoplasmosis. Although our study excluded type III *T. gondii* as a leading cause of abortion in humans. Nevertheless, it remains uncertain to what extent the genotype of the parasite directly contributes to the clinical severity of human toxoplasmosis. Further studies are highly recommended using larger number of samples, especially in placental toxoplasmosis where treatment might be enhanced.

Conflict of interests: The authors declare that they have no conflict of interest

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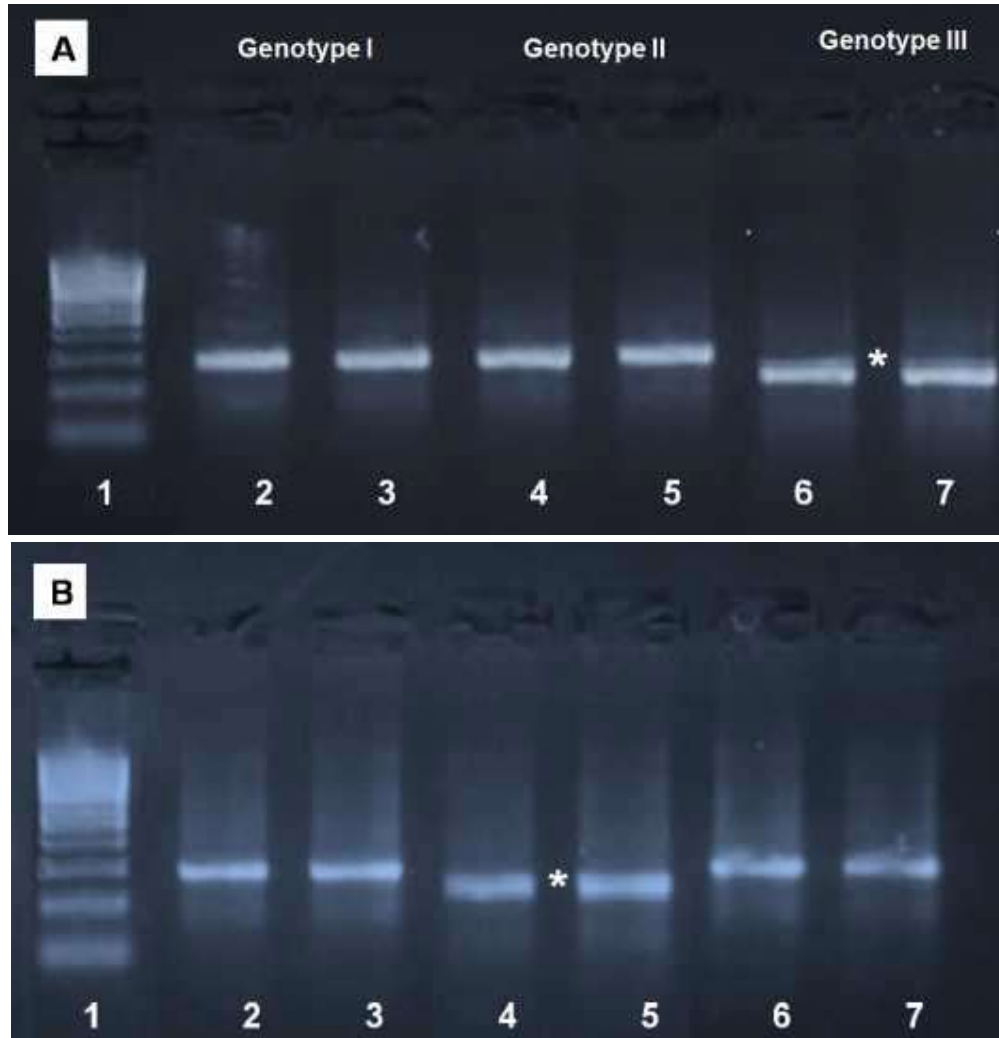


Figure (1.A): *SAG2* PCR amplification products of *Toxoplasma* genomic materials analyzed by agarose gel electrophoresis and *Sau3AI* restriction analysis of 5' amplification products. First lane DNA marker with 100 bp ladder. *Toxoplasma* genotype III (lane 6 & 7) only type digested with *Sau3AI* restriction enzyme (\*represented by 221bp band). Type I (lane 2 & 3) and type II (lane 4&5) not digested by *Sau3AI* restriction enzymes. Fig. (1.B): *SAG2* PCR amplification products of *Toxoplasma* genomic materials analyzed by agarose gel electrophoresis and *HhaI* restriction analysis of 3' amplification products. First lane DNA marker with 100 bp ladder. *Toxoplasma* genotype II (lane 3 & 4) only type digested with *Sau3AI* restriction enzyme (\*represented by the 221bp band). Type III (lane 5 & 6) not digested. Type I (lane 1 & 2) not digested by both *HhaI* and *Sau3AI* restriction enzymes