Genotyping of *Toxoplasma gondii* strains isolated from patients and mice by PCR-RFLP assay

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Abstract

In order to determine the different genotypes of *Toxoplasma gondii* strains, genetic analysis of SAG2 locus by PCR-RFLP assay was performed for the first time in Iran. Twenty-one isolates obtained from *Toxoplasma* infected human and mice from Iran (Tehran and Lajijan) were used for molecular typing. None of the isolates had any passage in mice. Genotype determination was made directly on samples. Type II was the predominant lineage, accounting for 85.7% of the isolates. Type I was found in 14.3% of cases with more presentation in animal samples, whereas type III lineage was not seen. No correlation was found between genotype and source of sample (blood, CSF and amniotic fluid). This study indicates a higher prevalence of type II lineage of *T. gondii* in infected human and mice in the above mentioned regions of Iran. 

Keywords: *Toxoplasma gondii*, SAG2 locus, RFLP.

INTRODUCTION

The protozoan *Toxoplasma gondii* (*T. gondii*) is the prevalent parasite responsible for widespread toxoplasmosis among warm blooded vertebrates (Howe and Sibley, 1995). The genus *Toxoplasma* has only one species, known as *gondii* with different strains (Binas and Johnson, 1998), which have been classified according to their biological differences (Literac et al., 1998) and virulence in mice. Some studies on genetic variability of these strains have been employed with different approaches including: antigen analysis (Bohne et al., 1993), isoenzyme assays (Darde et al., 1992; Asai et al., 1995), randomly amplified polymorphic DNA (RAPD), PCR detection (Guo et al., 1997) and restriction fragment length polymorphism (RFLP) (Christina et al., 1991; Christina et al., 1995; Howe et al., 1994; Sibley et al., 1992; Parmely et al., 1994).

*T. gondii* prevalence in Iran have been shows to be up to 50% which increases from dry to humid provinces in north of Iran (Assmar et al., 1997). The aim of this study was to determine the genotypes of *T. gondii* isolates obtained from clinical and animal sources in different parts of Iran. In this study we used RFLP assay in order to analyze the SAG2 locus “encoding a surface tachyzoite protein, called P₂₂” originally described by Howe et al., (1997). For primary detection of Toxoplasmosis in addition to screening and selection of positive specimens for genotyping, PCR using *B1* gene as a target sequence was also applied (Burg et al., 1989).

MATERIALS AND METHODS

Sources of *Toxoplasma* isolates: Two hundred specimens were obtained from patients referred to the Department of Parasitology at the Pasteur Institute of Iran in a period between 1998-2000. Clinical sources of the specimens included cerebrospinal fluid (10 samples), blood (40 samples) and amniotic fluid (150 samples). Animal specimens consisted of 145 samples of wild type mice belonging to two geographic
locations, Tehran (45 samples) and the Lahijan (100 samples) were randomly selected. Presence of infection in the samples were detected by \textit{B1} nested PCR method (Khodai et al., 1988; Burg et al., 1989).

**Chromosomal DNA extraction:** For DNA extraction from blood, cerebrospinal fluid and amniotic fluid specimens, 0.5 ml of each sample was added to 1 ml of lysis buffer (0.3 M sucrose, 10 mM tris base, 5 mM MgCl$_2$, 1% Triton X-100, 50 mM NaOH, 1 mM Tris HCl, pH 7.5). After centrifugation (5500×g, 1 min, 25°C) the sediment was mixed with 100 µl of 50 mM NaOH. The tubes were kept in boiling water bath for 20 min, 20 µl of 1 M Tris-HCl was then added to each tube, then mixed and centrifuged again (5500×g, 1 min, 25°C). The supernatant was transferred into a new tube and stored at -20°C as a template for PCR application (Jackson et al., 1993).

DNA samples from mice brain tissues were extracted according to the following instruction: 0.5 ml of homogenised mouse brain tissue was mixed with 0.5 ml of lysis buffer [SDS 1% and 10 µl of proteinase K (100 µg/ml)] and incubated at 55°C for 1 h. The lysates were repeatedly extracted with equal volume of phenol chloroform until the upper phase was free from contaminating proteins. After ethanol precipitation, DNA was dissolved in 20 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and kept at -20°C (Jackson et al., 1993).

Detection of \textit{Toxoplasma gondii} infected samples by nested-PCR: Specific primers were obtained from Cina gene (Iran) and PCR amplification on \textit{B1} gene was performed as described by Burg et al. (1989). Briefly, the extracted DNA (from above mentioned samples) was used as template for first PCR reaction to amplify the region corresponding to 572-1160. In the second set of the reaction the product of the first reaction was used as template to amplify a fragment corresponding to 694 to 853 (Khodai et al., 1988 and Burg et al., 1989).

Amplification of \textit{SAG2} locus as a target for genetic polymorphism analysis: \textit{SAG2} locus has two polymorphic sites at 3’ and 5’ ends for type II and type III (Howe and Sibley, 1997) so, amplification of this locus at either end was performed separately. The 5’ end of \textit{SAG2} locus was amplified by standard PCR with specific outer primers as follows:

- **Sense:** F3 (5’-TCTGTTTCTCCGAAGTGACTCC- 3’)
- **Antisense:** R3 (5’-TCAAAGCGTGCATTATCGC-3’)

One microliter of PCR amplicon was subsequently used as a template for Nested-PCR with specific inner primers as following:

- **Sense:** F2 (5’-ATTCTCATGCCTCCGCTTC-3’)
- **Antisense:** R (5’-AACGTTCACGAAGGCACAC-3’)

The protocol for temperature cycling included 30 cycles that consisted of 1 min of denaturation at 93°C, 1 min at the annealing temperature of 63°C, and 1.5 min of extension at 72°C. The final extension step continued for an additional 5 min. The 3’ end of \textit{SAG2} locus was similarly amplified by standard PCR with specific primers:

- **Sense:** F4 (5’-GACCTCGAACAGGAACAC-3’)
- **Antisense:** R4 (5’-GCATCAACAGTCTCTGTTGC-3’)

One microliter of this PCR product was directly used for nested-PCR as a target with primers mentioned here:

- **Sense:** F (5’-GAAATGTTTCAGGTTGCTGC-3’)
- **Antisense:** R2 (5’-GCAAGAGCGAACTTGAACAG-3’)

The protocol for temperature cycling was used as described for the 5’ end of \textit{SAG2} locus, except for the annealing temperature (65°C was applied for the 3’ end of the gene). The reaction mixture (30 µl) contained 670 µM Tris HCl, pH 8.8, 160 µM (NH$_4$)$_2$SO$_4$, 3 mM MgCl$_2$, 0.1% Tween-20, 200 µM of each dNTPs, 1 µg of each primer pair, and one unit of Taq polymerase (Biotaq, Russia). The reaction mixture was covered with 100 µl of mineral oil. Amplifications were performed in a thermal cycler (Pharmacia, UK).

PCR process at \textit{B1} and \textit{SAG2} loci, was performed on two control groups viz, non-infected mouse brain and distilled water to check possible contaminations. Products were electrophoresed on 1.2% agarose gel, stained with 0.5 µl of ethidium bromide and then visualized under UV illuminator.

**RFLP:** Five microliter of the nested-PCR products of the 3’ and 5’ ends were digested using 5 U of each \textit{HhaI} and \textit{Sau3AI} restriction enzymes (Boehringer, German) respectively in separate reactions in a total volume of 20 µl at 37°C. All digestions were performed according to the manufacture’s instructions.

**RESULTS**

In the present study, a total of 345 clinical specimens from 200 patients and 145 mouse brain tissues were obtained from Tehran and Lahijan cities of Iran. They
Roodabe Behzadi et al. were tested for detection of the B1 gene of *T. gondii* by nested-PCR assay (Burg et al., 1989).

Among these specimens, 21 samples were found positive for the presence of *T. gondii* parasite. They were further analyzed for genotype determination by Nested-PCR-RFLP at SAG2 locus. PCR reactions on 3’ and 5’ ends resulted in a 221 bp and a 241 bp amplified bands respectively (Fig. 1). Which were consistent with those of a previous report (Howe et al., 1997).

On the basis of the alleles identified at SAG2 locus, *Toxoplasma* isolates have been grouped into three lineages (Howe et al., 1997; Mondragon et al., 1998). Type I is determined by resistance of the 3’ and 5’ end nested product of the SAG2 locus to cleavage by *HhaI* and *Sau3AI* respectively. Resistance of 5’ end of SAG2 locus to cleavage by *HhaI* determines type II (Fig. 2). Type III is determined by resistance of the 3’ end Nested-PCR products of SAG2 locus to cleavage by *Sau3AI* (Fig. 2). Of a total of 21 strains investigated here, type II was the most prevalent genotype accounting for 85.7% of the isolates (Table 1). Type I was found in only 14.3% of the isolates, whereas genotype III was not found at all. Moreover, in our study, there was no correlation between the determined genotypes and the sources of samples (blood, CSF and amniotic fluid). Manufacturer protocol and positive controls confirmed the appropriate activity of the restriction used enzymes.

**DISCUSSION**

In this study, we used a rapid and efficient procedure for genotyping 21 *T. gondii* strains isolated from human and mouse populations in Iran based on PCR-RFLP assay at SAG2 locus. Previously, the TGR1E genomic sequence had been exploited as a target for molecular genotyping of *T. gondii* strains (Christina et al., 1991, 1995). Other investigators have used IC intron found within the DNA polymerase α gene of *T. gondii* for genotype determination. (Sibley and Boothroyd, 1992). In addition the intron of beta tubulin gene for the aim of genotyping was investigated by Costa and Darde (1997). All of these studies have resulted in identification of only two clonal lineages in *T. gondii* population. SAG1 locus encoding a stage specific protein, named P30 was also used as a target for molecular genotyping of *T. gondii* isolates, but was found not to be polymorphic enough to determine the strain genotype (Burg et al., 1988). PCR-RFLP assay...
at SAG2 locus demonstrated a three clonal lineage in *T. gondii* population with RFLP variations being identified by *HhaI* and *Sau3AI* restriction endonucleases (Mondragon et al., 1988 and Howe et al., 1997 and Honore et al., 2000 and Funes et al., 2001). In previous studies, type II was found to be the most prevalent lineage accounting for 80%, 83.3% and 76.7% of their isolates, (Howe et al., 1997; Mondragon et al., 1998; and Honore et al., 2000) respectively.

The frequency of lineage II in this study was 85.7%, which in accordance with previous reports and confirms that type II strains are associated with the majority cases of toxoplasmosis (Mondragon et al., 1988 and Howe et al., 1997 and Honore et al., 2000). In contrast to the above results, Funes et al., (2001) reported that type II strains were not associated with all forms of toxoplasmosis. They found that type I was the most prevalent lineage in Spain containing about 75% of congenital toxoplasma infections. They have suggested that these differences might be affected by selection in the process of culture and isolation of the sample before genetic characterisation (Funes et al., 2001).

In the present study, type I was found in 14.3% of isolates which all obtained from noncongenital cases, whereas, all of the congenital isolates belonged to type II, which is similar to the results of Howe et al., (1997). It is reported that type I strains are often lethal in mice (Derouin et al., 1991; Sumyuen et al., 1995). In addition, according to the data in table 1, the frequency of type II strains in nature (animal population) is lower than the frequency of this type in human cases. Therefore this may be an association between the high prevalence of type II and toxoplasmosis in human, it could be due to the type of diet and its differences between human (omnivorous) and mice (herbivorous).

In this study, we did not find any type III isolate, whereas in pervious reports it has been determined to be about 7.7% (Mondragon et al., 1998; Honore et al., 2000). The reasons for such an observation could be due to the limitations both, for the sample size and geographic locations in our study.

To confirm the presence or absence of type III lineage, a larger number of isolates from the studied areas are required.

In summary, we reported here the genotyping results of 21 *T. gondii* isolates from Iran based on PCR-RFLP assay at SAG2 locus. According to the previous studies (Howe et al., 1997; Mondragon et al., 1998; Honore et al., 2000), which emphasized the prevalence of type II lineage, our results also indicated that this type of *T. gondii* is more prevalent than type I in Iran. However, more studies are required to clarify the differences in the pathogeneity of these types. Finally, results of our study suggest that future investigations on the relationship between *T. gondii* genotypes and their pathogeneity, should emphasis more on type II *T. gondii*.

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**References**


