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Original Article

Genotyping of *Toxoplasma gondii* Strains Isolated from Patients with Ocular Toxoplasmosis in Iran

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Received 10 Dec 2015
Accepted 22 Jun 2016

Keywords:
Genotyping,
Toxoplasma gondii,
Ocular toxoplasmosis,
GRA6,
PCR-RFLP,
Iran

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Abstract

Background: *Toxoplasma gondii* is an obligate, intracellular protozoon that develops its sexual stage in cat's intestinal epithelial cells as definitive host and develops its asexual stage in different tissues of a wide range hosts called intermediate host. The protozoon is a food-borne and worldwide parasite that can cause serious complications such as abortion in pregnant women, encephalitis, and ocular toxoplasmosis. The present study aimed to genotype *T. gondii* strains isolated from patients with toxoplasmic retinochoroiditis.

Methods: Fifty-two blood samples were taken from patients with ocular toxoplasmosis, from July 2013 to July 2014. The specimens were collected from three ophthalmological hospitals of Tehran, Iran. After that, DNA extraction was performed using kit on separated buffy coats of serologically positive blood samples. Then PCR was done in GRA6 gene. For digestion of products, *MseI* endonuclease was used. Finally, some of the PCR products were sequenced.

Results: All of 52 samples were found positive by serological and PCR-RFLP methods and all of isolated strains belong to type III genotype. Type III genotype has the highest prevalence in Iranian ocular toxoplasmic patients.

Conclusion: *T. gondii*, particularly its type III should not be neglected as a cause of retinochoroiditis.

Introduction

T. gondii is an intracellular parasitic protozoan that belongs to the phylum Apicomplexa with a worldwide distribution and a wide range of hosts including warm-blooded animals such as human, livestock, birds, and marine mammals (1-5). The parasite has a complex life cycle involving so that asexual form develops in various tissues of intermediate hosts and sexual form develops in epithelial cells of cat gut (1). Human infection generally occurs by consuming tissue cysts in raw or undercooked contaminated meat or by ingestion of oocysts shed in the infected cat feces (6).

It is estimated that 15%-85% of human population around the world are chronically infected with toxoplasmosis and up to 50% of human population are infected in Iran (7). In spite of the fact that most cases of human infection are without clinical manifestation or mild, the parasite can cause serious complications such as encephalitis in congenitally infected children and immunocompromised patients, but retinochoroiditis occurs often in individuals with normal immune system (8, 9).

Ocular toxoplasmosis is one of the main causes of posterior uveitis around the world. Clinical presentation, relapse rate, and severity of ocular lesions depend on various factors, such as geographic location, host's immune status, time of infection, and exposure rate. One of the important factors that can influence parasite virulence including ocular toxoplasmosis is its genetic makeup. Thus, assessment of genetic diversity among isolates that are responsible for disease must be accomplished. According to early different molecular methods *T. gondii* has three main genotype including I, II, and III and some minor and mixed genotypes but recent studies revealed that the parasite is genetically diverse (10-14). Three main clonal lineages of the parasite have less than 1% difference in their genetic structure (15). In Europe, researchers have defined more than 95% of *T. gondii* population belong to one of the types I, II, and III, but a different population structure has been found in other regions (16, 17).

For detection of toxoplasmosis serological methods such as ELISA, sabin feldman dye test and molecular methods can be employed. There are several genetic markers to identify genotypes of *T. gondii* isolates such as surface antigens including SAG1 to SAG4, MAG1, BSR4 and SRS1 to 3, and excretory secretory antigens including GRA1 to GRA4, GRA6 and ROP1. One of the most famous marker is dense granule antigens, named GRA proteins, which mainly expressed within tachyzoite (18, 19), but also expressed within encysted stage or bradyzoite, too. Probably these molecules play role in intracellular survival by changing host cell mechanisms. GRAs are mitochondrial gene. This protein family has 15 member, including GRA1 to GRA15. GRA1, found in parasitophorous vacuole (PV) is a calcium binding protein (20-22). GRA2 and GRA4 are firmly linked to the membranous network of PV by linking to lipids and transmembrane region, respectively (21, 23-25). GRA3 and GRA5 are distributed on the membrane of the PV (23, 26, 27). GRA6, used to identify the parasite in our study, is a 32 kDa member of GRA family that has antigenic cross-reactivity with GRA5 protein (28). GRA5 be detected in the dense granule of tachyzoite and PV (28). Tere is only a single copy of GRA6 gene, which does not contain any introns, in the genome of *T. gondii*, and potentially encodes a 230-amino-acid polypeptide (28). Some of GRA proteins were used for immunodiagnosis of toxoplasmosis, for example Arab-Mazar eal. used GRA7 for this aim (29). In the previous studies some other loci such as SAG1, GRA4, and β-tubuline gens have been used to genotyping *T. gondii* but they have less polymorphism and they cannot distinguish type II and III (30-32). Also some researchers tried to

clone GRA proteins to produce recombinant GRA proteins or use them as DNA vaccines (33).

The present study aimed to genotype *T. gondii* strains isolated from patients with toxoplasmic retinochoroiditis by using GRA gene.

Materials and Methods

Sampling

A total of 52 blood samples were taken from patients with suspected active ocular toxoplasmosis from three ophthalmological hospital of Tehran, Iran, from July 2013 to July 2014. Patients were from different regions and cities of Iran. Before sampling ophthalmologists confirmed ocular toxoplasmosis of suspected individuals through ophthalmoscopic examination. Vitreous humor samples for detection of ocular toxoplasmosis are better but sampling of vitreous humor is invasive, so blood samples were collected to accomplish this study.

DNA extraction

All of the samples (52 samples) were assayed by serological method (ELISA, Acone kit) and serologically positive samples (all of 52 samples were found positive including 3 IgM positive and 49 IgG positive) were selected for DNA extraction. To extract DNA at first buffy coats of samples were separated. After that DNA were extracted from buffy coats using kit (QIAGEN Company). DNA was extracted by the instruction of the company. Extracted DNA was kept at -20 °C.

PCR amplification

Extracted DNA was amplified using GRA6 primer (34). Primer pairs consisted of GRA forward primer (5`-GTAGCGTGCTTGTTGGCGAC-3') and GRA reverse primer (5`-TACAAGACATAGAGTGCCCC - 3') (34). An approximately 791bp can be amplified by these primers. PCR was performed in a ready-made mixture ampliqon (Taq DNA Polymerase Master Mix, Denmark). The final mixture of the reaction contained 7.5 µL of Taq Master Mix (2X), 7 ng DNA, 10 pmol of each primer, and 4.5 µL distilled water. After preparing mixture of reaction, PCR was carried out in 33 cycles, and under following condition: initial denaturation step at 94°C for 5 min, denaturation step at 94 °C for 35 sec, annealing step at 56 °C for 60 sec, extension step at 72 °C for 30 sec, and final extension 72 °C for 10 min. For conformation of PCR results, its products were analysed using 1.5% agarose gel (for separation), staining with a solution of ethidium bromide, and examination under UV light.

PCR-RFLP examination

To determine the parasite strain PCR-RFLP was performed on PCR products of positive samples. First, *MseI* endonuclease was used for digestion of products. This restriction enzyme distinguishes different types of parasites by cutting products to 168 and 544 bp, 75 and 623 bp and 97 and 544 bp fragments in type I, type II and type III, respectively (34). After adding the enzyme, product and enzyme mixture were incubated at 37 °C for 4 h to cut GRA6 gene by the enzyme function. Finally, staining and examination under UV light were performed to conformation of function of the enzyme and determination of the parasite strain separation.

Sequencing of PCR products

To verify the *T. gondii* genotypes, randomly 5 purified PCR products from positive samples were submitted for sequencing using ABI 3130X automatic sequencer at the Bioneer Company, South Korea. In order to classify the isolates, BLAST program of the US National Center for Biotechnology Information (NCBI) site was used to carry out analysis of homology of obtained sequences with submitted genes in gene bank.

Maximum likelihood (ML) tree was constructed via MEGA v5.05 for showing the phylogenetic position of the GRA6 sequences based on the Kimura 2-parameter model of

nucleotide substitution search by stepwise addition of 100 random replicates and bootstrap values with 1000 replicates.

Ethical considerations

This study was conducted under Shahid Beheshti University of Medical Sciences Ethics Committee approval and written consent of patients was received.

Results

All of 52 serologically positive samples were positive for *T. gondii* DNA through amplifying GRA6 gene using PCR method, and 791 bp bands were detected under UV light after transferring PCR products to 1.5% agarose gel (Fig. 1). In addition, *MseI* endonuclease (to carry out PCR-RFLP) was added to all of positive samples. After carrying out PCR-RFLP 544 bp and 97 bp bands were detected under UV light, small bands are not visible (Fig. 2). These bands revealed that all of our isolates belonged to type III allele of GRA6 of *T. gondii*. Analysis of 5 sequenced products verified that our isolates belonged to type III which three (sequencing results of three extracted DNAs were same) of sequences are available in Genbank with accession numbers: Kt159894, Kt362355 and Kt362656. Homology of our sequences compared with genebank sequences (Fig. 3). Figure 4 shows a phylogenic tree for GRA6 nucleotide sequences of our 3 isolates, together with 14 reference strains of *T. gondii* and one strain of a *Hammondia hamondi*.

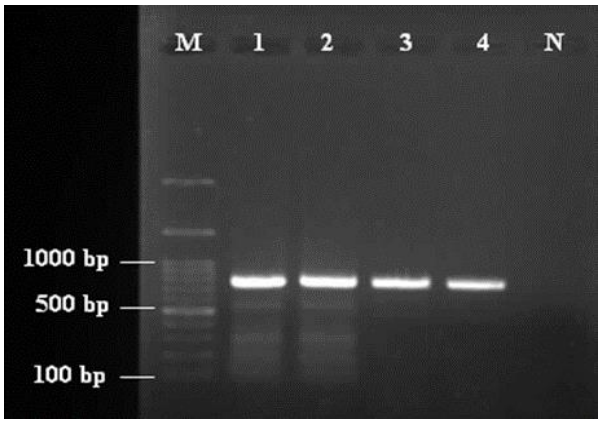

Fig.1: Agarose (1.5%) gel showing the PCR products (791bp) of amplified from positive samples. Lane M is DNA size marker, lanes 1-4 are positive samples and lane N is negative control

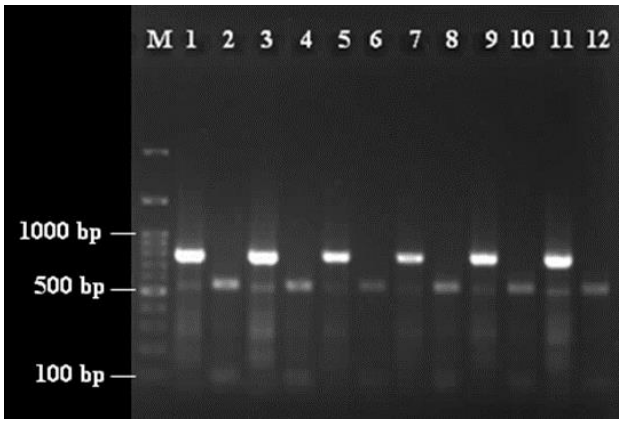

Fig. 2: Agarose (1.5%) gel showing the PCR-RFLP pattern of GRA6 gene cut with *MseI* endonuclease (544 bp and 97 bp bands). Lane M indicating DNA size marker, lanes 1, 3, 5, 7. 9 and 11 uncut PCR products before using enzyme, lanes 2, 4, 6, 8, 10 and 12 RFLP pattern of type III *T. gondii*

Discussion

In the present study, we used PCR-RFLP assay at GRA6 locus for genotyping of 52 DNA of *T. gondii* strains isolated from ocular toxoplasmic patients. There are various studies that considered genotyping of *T. gondii* isolates from immunodeficient patients, congenital toxoplasmosis cases, soil or animals (13, 35), and also some studies (with less frequency in comparison with previous studies) considered this aim in symptomatic acquired toxoplasmosis in patients with normal immune system (36-38).

To the best of our knowledge, there is no study on genotyping of *T. gondii* isolates from ocular toxoplasmosis patient using PCR-RFLP methods, in Iran.

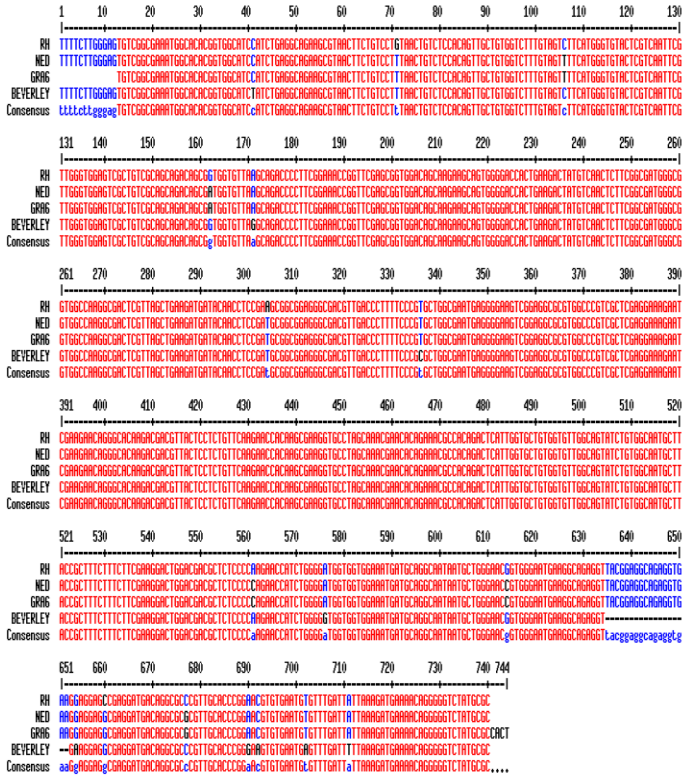

Fig. 3: The comparison of sequencing of GRA6 gene isolated from patients with gene bank sequences

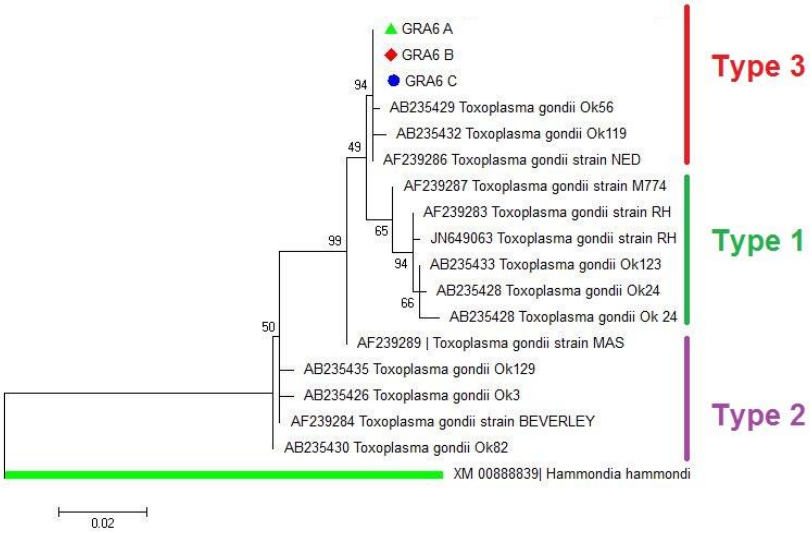

Fig. 4: The phylogenic tree was constructed by maximum likelihood method using the nucleotide sequence of reference strains and our isolates (indicated with colorful shapes behind them). The scale bar indicates a 2% nucleotide difference

In this study, GRA6 was used for genotyping the parasite because the coding region of this gene has considerable polymorphism, and even in comparison to other examined *T.* cod-

ing genes such as SAG1, SAG2, and GRA4 is more variable (30, 31, 39). Rate of amino acid changes, non-synonymous to synonymous, is high so this fact show variation in GRA6 genes of different isolates of *T. gondii* may influence survival of the parasite particularly in the parasitophorous vacuole (34).

In the both of immunocompetent and immunocompromised patients ocular toxoplasmosis is one of the most common causative agents of infectious retinitis (40). According to host genetic and genotype of parasite, severity of disease and clinical manifestation can present variations (41). Although type II is predominant in human toxoplasmosis (17), type I was considered as most virulent type. In comparison with genotype II genotypes I and recombinant of I and III are more common causative agents of severe human ocular toxoplasmosis (36), in our study all of the isolates (52 isolates) were detected type III, whereas type I and type II was not found.

Some studies around the world were accomplished to find out which type of *T. gondii* is responsible for ocular toxoplasmosis. Switaj K. et al. investigated relationship between ocular form of toxoplasmosis and type I of the parasite, as the most virulent type of the parasite in mouse (42). In this study like our study whole blood samples were used for genotyping of the parasite instead of vitreous humor because taking blood sample is safe, convenient and useful (42). In one study researchers detected genetic material of the parasite in 86.9% (53 out of 63) of patients with active retinochoroiditis, and also in 50% (6 out of 12) of patients with old scar by PCR method. Another study that was conducted in Brazil using SAG1 locus, revealed that all 11 strains causing retinochoroiditis belong to type 1 (43). In USA, type I and recombinant genotypes with a type I are predominant (36).

Ferreira et al. used PCR-RFLP method on several genetic markers including SAG1, SAG2, 5´- and 3´-SAG2, alt.SAG2, SAG3, BTUB, GRA6, C22-8, c29-2, L358, PK1, and Apico (44). In this study, detected parasites were grouped in three distinct groups.

In one study researchers determined genotype of *T. gondii* using PCR-RFP on GRA6 gene, they used samples from different hosts (including human, sheep, rabbit, guinea pig, cat, chicken and monkey) with different clinical signs and different countries (including USA, New Zealand, France, Germany, Scotland, England and French Guyana) detected type I and type II, unlike our study type III was not found in their study (34).

GRA6 was used to genotype animal isolates of *T. gondii*. Biradar et al. characterized isolates from chicken using GRA6 gene sequence analysis in India (45). They reported that chicken isolates of the parasite belong to type III.

Prior to our study, in several studies genotyping of various isolates of *Toxoplamsa gondii* was carried out but to the best of our knowledge in the present study for the first time samples were collected from ocular toxoplasmosis patients in Iran. Rashidi et al. carried out molecular characterization of the parasite from rats in Tehran using PCR and sequencing; according to alignment results their isolates had had the highest similarity (81-95%) with RH stain (46). They found 50% of rats (20 out of 40) positive and their isolates had the most similarity with RH strain.

Conclusion

T. gondii, particularly its type III plays an important role in retinochoroiditis occurrence in Iran and it should not be neglected by ophthalmologists.

Acknowledgments

We would like to thank to Dr. Niloofar Taghipour for her kind cooperation. The authors would like to thank all patients for giving permission to collect samples and participated in this study. We are thankful to the

Protozoology Unit of the Shahid Beheshti University of Medical Sciences for provision of materials. The authors declare that there is no conflict of interest.

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