

Ocular Toxoplasmosis in Benghazi, Libya: Evidence of Type I Strain

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Abstract: *Toxoplasma gondii* a tissue protozoan affecting one third of humanity has subpopulation structures in different geographical regions caused by less frequent sexual recombination and population sweeps. The majority of strains isolated in North America and Europe fall into one of three clonal lineages, referred to as types I, II, and III. So far, little is known about *Toxoplasma* strains in Africa. Ocular toxoplasmosis is an important complication of *T. gondii* infection in the immunocompetent. The present study aimed to determine the genotype of *Toxoplasma* strains from ocular toxoplasmosis patients in Benghazi, Libya. Blood from 43 patients clinically suspected of ocular toxoplasmosis were obtained and serologically screened for anti-*Toxoplasma* antibodies. 24 (55.8%) seropositive (IgG) samples were subjected to PCR to obtain *T. gondii* DNA. Only 6 (25%) samples had enough DNA and were genotyped through single locus nested-PCR-RFLP analysis based upon the SAG2 gene. The restriction pattern revealed the presence of genotype I in all samples. This is the first genotypic evidence (type I strain) based on single locus PCR-RFLP analysis for ocular toxoplasmosis cases from Benghazi, Libya.

Keywords: Ocular toxoplasmosis, PCR-RFLP, *T. gondii*

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I. Introduction

T. gondii is an obligate intracellular protozoan of the phylum Apicomplexa infecting birds and mammals. It is estimated that one-third of global population is infected with *T. gondii* (Robert-Gangneux & Darde 2012). Infection in the immunocompromised can be fatal but largely asymptomatic in the immunocompetent. Transmission dynamics and virulence however have been linked to genotypic strains. *T. gondii* isolated from North America and Europe belongs to one of three clonal lineages, which are referred to types I-III (Howe & Sibley, 1995) with type II strain showing increasing link to virulence (Peyron et al. 2006). However atypical strains have been linked to endemic areas of Central and South America (Rajendran et al. 2012). Although genetic differences amongst strains are only about 1-2% (Weilhammer & Rasley 2011), virulence differs significantly. Ocular toxoplasmosis is an important congenital complication in the immunocompetent. Type I strain which is a virulent strain in mice have been linked to ocular toxoplasmosis (Grigg et al 2001; Vallochi et al. 2005). Several methods have been used to strain type *T. gondii* which include isoenzyme electrophoresis (Dardé et al. 1992), DNA sequencing (Fazaeli et al. 2000), DNA microsatellite analysis (Ajzenberg et al. 2002), single (Vallochi et al. 2005) and multi locus (Su et al. 2006) PCR-RFLP (restriction fragment length polymorphism) and multiplex PCR (Nowakowska et al. 2006). However, PCR-RFLP is the most common method used in strain typing many isolates in the world and had produced significant data on *T. gondii* genetic variation (Shwab et al. 2014). Many genetic markers have been identified and used in strain typing *T. gondii* isolates but parasite surface antigen genes like SAG2 is widely used in RFLP analyses (Vallochi et al. 2005; Su et al. 2006). This study reports the genotype of *T. gondii* among ocular toxoplasmosis patients from Benghazi, Libya.

II. Material And Methods

Sample collection

Venous blood sample from consenting adult patients (≥ 18 yrs) who were not HIV positive or having diabetic retinopathy and suspected of ocular toxoplasmosis from clinical examination (Commodaro et al. 2009) presenting to the ophthalmology clinic at Sohael Al-Atrash Eye Hospital, Benghazi, Libya between January 2013 and July 2015 were used.

Ethical approval

Ethical approval number NMRR-14-1655-21207 was obtained from the Malaysian Ministry of Health and institutional clearance was approved by the University of Benghazi.

Serological screening

Serology screening for anti-Toxoplasma antibody (IgG and IgM) was performed using standard ELISA kit (EUROIMMUN, Lübeck, Germany) according to manufacturer's instruction.

PCR

DNA was extracted from serum samples using QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands) according to manufacturer's instruction. To obtain enough DNA material, nested PCR was performed targeting Toxoplasma B1 gene (Alfonso et al. 2008).

Genotype analysis

Samples positive for *T. gondii* DNA were subjected to genotypic analysis for detecting the prevalent genotype infecting these cases. Genotyping was based on first amplifying the 5' and 3' ends of the SAG2 gene in nested PCR reactions. Amplified products were subjected to restriction digestion reactions that produce a certain restriction pattern corresponding to a certain specific genotype. For distinguishing allele 3 (genotype III strains) from alleles 1 and 2 (genotype I and II strains), the 5' end of the SAG2 was analyzed. For amplifying the 5' end of the SAG2 gene, the following primer pairs were used SAG2.F4 (5'-GCTACCTCGAACAGGAACAC-3') and SAG2.R4 (5'-GCATCAACAGTCTTCGTTGC-3'). Cycling conditions consisted of an initial denaturation step of 1 min at 95°C followed by 40x cycles of 30s denaturation step at 95°C, 1min annealing temperature of 65°C and 1 min extension at 72°C and a final extension step for an additional 5 min. Reactions were carried out using the Applied Biosystems (Massachusetts, USA) Verti™ thermal cycler machine. Following the first amplification product, the reaction mixture was diluted 1:10 in sterile water and 1µl of the diluted product was used as a template for the second PCR reaction. The nested PCR reaction was carried out using the internal primers SAG2.F (5'-GAAATGTTTCAGGTTGCTGC-3') and SAG2.R2 (5'-GCAAGAGCGAAGTTGAACAC-3'). Similarly, the reaction consisted of 40 cycles and annealing temperature of 65°C was employed to produce an amplified product of 241bp. Finally, products of the second amplification reaction were purified with PureLink™ PCR Purification Kits (Thermo Fisher Scientific, Massachusetts, USA) and then digested with Sau3AI restriction enzyme (Thermo Fisher Scientific, Massachusetts, USA). Digestion reaction was carried out in 50µl final volume and the reaction mixture was incubated for one hour at 37°C then the restriction fragments were separated by 2% agarose gel electrophoresis. Only genotype III strains were expected to be digested with the Sau3AI restriction reaction to produce 2 digestion products. Types I and II strains will not be affected by this enzymatic digestion. The same principle was employed to distinguish type II strain. However in this case the 3' end of the SAG2 was analyzed. Briefly, the 3' end of the locus was first amplified using the primers SAG2.F3 (5'-TCTGTTCTCCGAAGTGACTCC-3') and SAG2.R3 (5'-TCAAAGCGTGCATTATCGC-3'). Similarly, amplified products were diluted 1:10 then used for the second round of amplifications using the internal primers SAG2.F2 (5'-ATTCTCATGCCTCCGCTTC-3') and SAG2.R (5'-AACGTTTCACGAAGG CACAC-3') to produce a 221bp product. The resulting amplicons were purified and then digested with HhaI enzyme (Thermo Fisher Scientific, Massachusetts, USA). Digested fragments were electrophoresed on 2 % agarose gel containing 0.5µg/ml ethidium bromide and visualized using UV transilluminator. Only type II strains were expected to be digested with the HhaI enzyme. Types I and III strains will not be affected by this enzymatic digestion. Finally, type I strain were identified by exclusion of neither being digested by Sau3AI nor HhaI enzymes.

Table 1: Summary of the nested PCR and digestion protocols for genotypic analysis.

Target	Type	Primers	Sequence	Restriction enzyme	Aim
5' end of SAG2	External primers	SAG2.F4	5'GCTACCTCGAACAGGAACAC3'	Sau3AI	Identify type III strain
		SAG2.R4	5'GCATCAACAGTCTTCGTTGC3		
	Internal primers	SAG2.F	5'GAAATGTTTCAGGTTGCTGC3'		
		SAG2.R2	5'GCAAGAGCGAAC TTGAACAC3'		
3' end of SAG2	External primers	SAG2.F3	5'TCTGTTCTCCGAAGTGACTCC3	HhaI	Identify type II strain
		SAG2.R3	5'TCAAAGCGTGCATTATCGC3		
	Internal primers	SAG2.F2	5'ATTCTCATGCCTCCGCTTC3		
		SAG2.R	5'AACGTTTCACGAAGG CACAC3'		

III. Result

Convenient tertiary hospital-based sampling from January 2013 to July 2015 (2.5 years) resulted in 43 clinically suspect ocular toxoplasmosis cases. Serological screening detected 24 (55.8%) seropositive samples out of 43 clinically suspect ocular toxoplasmosis patients. Nested PCR targeting Toxoplasma B1 gene detected 6 positive samples out of 24 seropositive samples (25.0%). Fig 1 shows *T. gondii* positive representative samples.

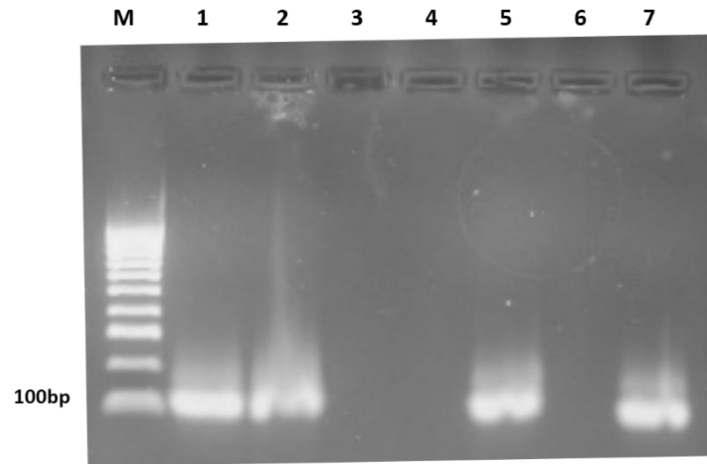
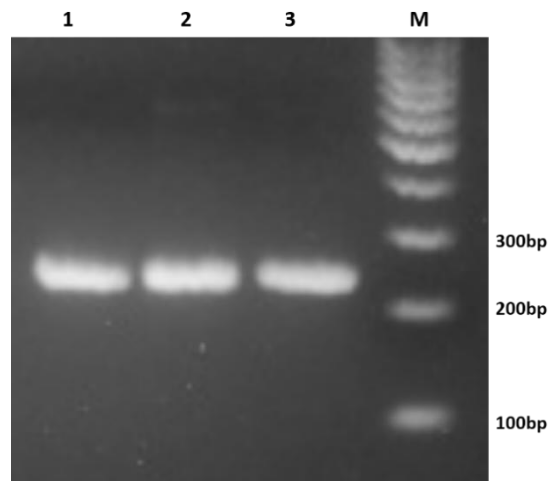


Figure no1. Gel electrophoresis image of nested PCR reaction for detection of *T. gondii* in serum samples. In lane M a 100bp ladder was added. Lanes 1, 2, 5, 7 showed amplified products that correspond to 97bp band. However, lanes 3, 4 and 6 were negative.

Genotypic analysis of samples was carried out by analyzing both the 5` and 3` ends of SAG2 gene by RFLP. First, samples were subjected to a nested PCR reaction to amplify a 241bp fragment of the 5` end. This fragment was then purified then digested with the *Sau3AI* enzyme. Fig 2 shows a representative sample of the analyzed samples. Lane 1 shows the nested amplified product that corresponds to the expected 241bp band, lane 2 shows the purified PCR product before digestion while lane 3 shows the product of the *Sau3AI* restriction digestion. It is clear that there was no effect for *Sau3AI* on the amplified 5` end product thereby excluding the possibility that the samples belong to type III strain. All analyzed samples showed the same restriction pattern.



Figureno 2. Analysis of the 5` SAG2 nested amplified product. Lane 1: nested amplified product; lane 2: purified non-digested sample and lane 3: sample after digestion with *Sau3AI* restriction enzyme and lane M: DNA ladder.

The second step in genotyping analysis involved amplification of the 3` end of SAG2 using a nested PCR reaction similar to that mentioned before for the 5`SAG2. An amplified product of 221bp was produced. This fragment was then purified and digested with the *HhaI* restriction enzyme. Fig 3 shows a representative sample of the analyzed samples. Lane 1 shows the nested amplified product that corresponds to the expected 221bp band, lane 2 shows the purified PCR product before digestion while lane 3 shows the PCR product after digestion with *HhaI*. There was no effect for of *HhaI* restriction enzyme on the amplified 3` end product thereby

excluding the possibility that the samples belong to type II strain. All analyzed samples showed the same restriction pattern. Since all nested products of 5' and 3' SAG2 amplifications were not affected by Sau3AI or HhaI restriction enzyme digestion, it can be concluded that all samples belong to genotype I.

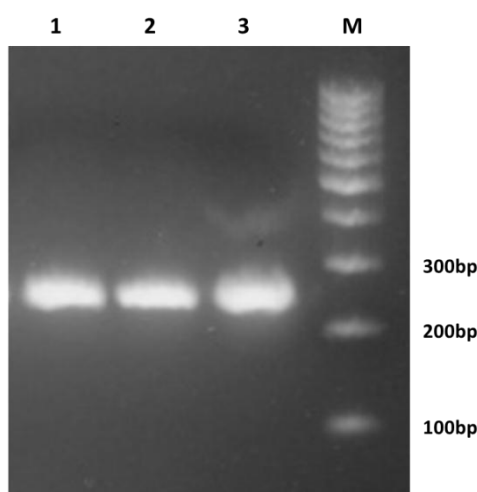


Figure no 3. Analysis of the 3' SAG2 nested amplified product. Lane 1: nested amplified product; lane 2: purified non-digested sample; lane 3: sample after digestion with HhaI restriction enzyme and lane M: DNA ladder.

IV. Discussion

Although our convenient sampling collection period was long (2.5 years), due to political instability in Libya, sampling could not be performed at most times which resulted in a small sample size of 43. However, similar tertiary hospital based data is comparable when only 31 ocular toxoplasmosis patients presented to a tertiary eye centre in Malaysia over a 1.5 year period (Lim and Tan 1983). The general population based prevalence of ocular toxoplasmosis is low. For example only 2% of the American population had ocular toxoplasmosis (Holland 2003). However, unusually high (17.7%) prevalence have also been recorded like in Brazil (Glasner et al. 1992). Out of the 43 clinically suspect cases only 24 (55.8%) were serologically positive (IgG). Although there are no Libyan data for ocular toxoplasmosis seroprevalence, study on pregnant women with complication recorded 44.8% IgG seropositivity (Mousa et al. 2011). Nested PCR detection of toxoplasma DNA targeting B1 gene (Alfonso et al 2008) only detected 6 samples (25%) from seropositive samples. Alfonso et al. 2009 recorded 75% sensitivity using CSF sample from AIDS patients. Ho-Yen et al. in 1992 recorded only 30.2% sensitivity from blood samples of AIDS patients. Our samples were sourced from non-AIDS patients. All toxoplasma DNA positive samples (6) were found to belong to type I strain by single locus (SAG2 gene) PCR-RFLP. Several methods have been used to strain type *T. gondii* which include isoenzyme electrophoresis (Dardé et al. 1992), DNA sequencing (Fazaeli et al. 2000), DNA microsatellite analysis (Ajzenberg et al. 2002), single (Vallochi et al. 2005) and multi locus (Su et al. 2006) restriction fragment length polymorphism (RFLP) and multiplex PCR (Nowakowska et al. 2006). However, PCR-RFLP is the most common method used in strain typing many isolates in the world and had produced significant data on *T. gondii* genetic variation (Shwab et al. 2014). We focused on a single gene for economical and practical reasons and SAG2 based genotyping has been shown to be specific due to the presence of polymorphic sites for each strain type (Mahalakshmi et al. 2010). Our results revealed all six samples belong to type I strain. This result adds to existing literature on the association of type I strain with ocular toxoplasmosis. An earlier study in Poland analyzing peripheral blood also found type I predominance (Switaj et al 2006) while another work using ocular fluid in Brazil also found only type I strain in all samples (Vallochi et al. 2005). Even multilocus analyses points to predominance of type I strain and other recombinant genotypes with type I allele (Grigg et al. 2001). Type I strain is the virulent strain in mice compared to types II and III. Type I strain has been demonstrated during outbreaks of ocular toxoplasmosis (Lehmann et al 2000) and a possible zoonotic link was seen in chickens (Dubey et al 2002) and cats (Dubey et al 2004) to ocular toxoplasmosis endemic areas in Brazil.

V. Conclusion

Ocular toxoplasmosis genotyping among Benghazi residents revealed type I strain. This study is the first toxoplasma genotypic study from Libya. Single locus genotyping however may not reveal atypical subtypes.

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