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Evaluation of Nested PCRs targeting the BI and SAG2 Genes for Detection of *Toxoplasma gondii* Genome in Aqueous Humor from HIV Positive *Toxoplasma* Retinochoroiditis Patients in a Tertiary Eye Hospital

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Abstract: Problem statement: To evaluate nPCR targeting B1 gene (primer set 1) with 3 other nPCRs targeting B1 gene (primer set 2), 3' end of SAG2 and 5' end of SAG2 gene, for detection of *Toxoplasma gondii* (*T. gondii*) genome on AH from HIV positive TRC patients. **Approach:** DNA extracted from AH of 12 TRC patients and 12 controls (patients with ocular inflammation of non-*Toxoplasma* origin) were subjected to all the 4 nPCRs. **Results:** *Toxoplasma gondii* genome was detected by atleast one of the 4 nPCRs in 8 (66%) of 12 TRC patients and in none of the 12 controls. nPCR targeting B1 gene (primer set 1) was positive in 6, B1 gene (primer set 2) in 2 and both 3'end of SAG2 and 5' end of SAG2 in 4 respectively. The sensitivity of B1 gene (primer I) was higher compared to the other 3 nPCRs (Yates correction-Chi square test; $p = 0.028$). **Conclusion:** Among the 4 nPCRs, nPCR targeting B1 gene (primer set 1) was the most sensitive and reliable nucleic acid amplification technique for the laboratory diagnosis of TRC in HIV patients.

Key words: *Toxoplasma* retinochoroiditis, B1 gene, HIV positive, polymerase chain reaction, SAG2 gene, aqueous humor

INTRODUCTION

Toxoplasma gondii (*T. gondii*), a protozoan parasite, can cause severe, life threatening disease, especially in newborns and immunosuppressed patients and is an important cause of ocular disease in both immunosuppressed and immunocompetent individuals (Holland, 1999). *T. gondii* may affect the brain, eye as well as the lung in case of individuals with AIDS/HIV, malignancy and organ transplant. *Toxoplasma* retinochoroiditis (TRC) is believed to account for 1-3% of retinal infections in patients with AIDS (Jabs *et al.,* 1989; Tabbara, 1990; Cochereau-Massin *et al*., 1992).

Early and appropriate treatment of ocular toxoplasmosis in AIDS patient is vital, because not only is the ocular prognosis good, but also because ocular disease often coexists with life threatening cerebral involvement and is the only presenting manifestation in 5% of these patients (Dupon *et al.,* 1995). Holland *et al*. (1988) have reported that in 25% of AIDS patients, TRC occurs in association with encephalitis. Jabs *et al*. (1989) have reported that 56% of patients with ocular disease also had cerebral involvement and 12% of patients with cerebral toxoplasmosis were found to have ocular disease as well. *Toxoplasma* encephalitis eventually develops in 25-50% of HIV patients with antibodies against *T. gondii* (Jabs *et al*., 1989; Tabbara, 1990; Cochereau-Massin *et al*., 1992). In these individuals the disease is fatal and can even cause death, if left untreated. Toxoplasmosis was the single most common aetiological agent (89%) of focal brain lesions at autopsy of 113 HIV patients in Bangalore, India compared to cerebral *Toxoplasma* lesions in 20 per cent of AIDS cases at autopsy in Mumbai, India (Shankar *et al.,* 2005). In a clinico-radiological study of 1527 HIV seropositive subjects from Pune, western India, by Wadia *et al*. (2001) toxoplasmosis was the commonest cause of mass lesions (66%).

There are several reports on application of PCR for detection of *T. gondii* in various clinical specimens like blood, cerebrospinal fluid, amniotic fluid, ocular fluids and tissue biopsy from immunocompetent and immuno suppressed patients (Dupon *et al.,* 1995; Aouizerate *et al.,* 1993; Garweg *et al.,* 2000; Bou *et al.,* 1999). The present study was to apply nested polymerase chain (nPCR) using primers targeting the B1 gene (Jones

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et al., 2000; Grigg *et al.,* 2001) and SAG2 gene (Fuentes *et al.,* 2001; Howe *et al.,* 1997) of *T. gondii* on aqueous humor (AH) and evaluate the diagnostic efficacy of these primers.

MATERIALS AND METHODS

Patients, controls and clinical specimens: The study protocol was performed in accordance with the Declaration of Helsinki. All patients and controls were recruited from Sankara Nethralaya, Chennai, India The study protocol was approved by the Ethics Committee of the institute and informed consent was obtained from each patient and control subjects. Twenty four AH, from 12 HIV positive patients with clinically suspected *Toxoplasma* Retinochoroiditis (TRC) and 12 controls were included in the study. The controls included HIV positive patients with etiology proven (by PCR) acute retinal necrosis (n = 6), CMV retinitis (n = 4) and *Mycobacterium tuberculosis* uveitis (n = 2).

Processing of Aqueous Humor (AH): AH samples (150- 200 μL) were collected aseptically in a tuberculin syringe with a 30-gauge needle, under aseptic precautions by the ophthalmologist and 50-100 μL of the sample was transferred into pre-sterilized microfuge tubes and stored at -20°C for DNA extraction.

DNA extraction: DNA was extracted from standard genotype I (RH strain) strain of *T. gondii* and 24 AH using DNA extraction kit (Biogene, USA) as per instructions given in the kit insert and was eluted in a final volume of 150 μL.

nPCR amplification: The nPCRs used in this study were standardized to detect *T. gondii* DNA. Their sensitivity was done by the standard protocol of ten fold dilution of the *T. gondii* DNA (RH strain), in Milli Q water and DNA concentration of the last dilution showing a positive result in nPCR is taken as the analytical sensitivity. The specificity was done using DNA extracted from bacteria (n = 6), fungus (n = 4), viruses (n = 3) and parasites-*Cysticercus cellulosae, Toxocara canis* and *Acanthameba* sps.

All primers and reagents for PCR were obtained from Sanmar Speciality Chemicals Limited, (formerly Bangalore Genei Pvt. Ltd.), Bangalore, India. Two reagent controls (one for the first round amplified in the second round also and another reagent control for the second round alone) and positive control (using 2 μL DNA from RH strain of *T. gondii*) were included in each PCR run. The PCR results were considered valid only when the reagent controls were negative and the specific amplified product was obtained with the positive control. To prevent contamination, DNA extraction, PCR preparation, PCR amplification and analysis of the amplified product were done in physically separated rooms. PCR preparation was performed on a laminar flow workbench with single use aliquots of reagents and dedicated pipettes. Sterile filter guarded tips were used for the addition of specimens and the extracted DNA. Visualization of PCR product was done by subjecting 10 μL of amplified reaction mixture to electrophoresis on a 2% agarose gel incorporating 5 μg mL^{-1} of ethidium bromide in 1X Tris-Borate buffer (pH -8.2-8.6) and documented on gel documentation system (Vilber Lourmat, France).

Positive control: DNA extracted from the tachyzoites of *T. gondii*-RH strain, (maintained in mice by Dr. M.L. Dubey, PGIMER, Chandigarh, India) was used as positive control for nPCR.

nPCR amplification of B1 gene (primer set 1): Nested primers targeting the B1 gene (Jones *et al.,* 2000) included outer sense primers for the first round of amplification consisting of upstream primer 5' GGA ACT GCA TCC GTT CAT GAG 3' and down stream primer 5' TCT TTA AAG CGT TCG TGG TC 3' which generates a 193 bp product (nucleotides 694-887). The inner set of II round of primers consisting of upstream primer 5' TGC ATA GGT TGC AGT CAC TG 3' and downstream primer 5' GGC GAC CAA TCT GCG AAT ACA CC 3' generate a 96 bp product (nucleotides 757-853) after amplification by nPCR. The nPCR reaction and the protocol were as given elsewhere (Mahalakshmi *et al.,* 2006).

nPCR amplification of B1 gene (primer set 2): Nested primers coding for the B1 gene (Grigg *et al.,* 2001) included outer sense primers for the first round of amplification consisting of upstream primer- 5'-TGT TCT GTC CTA TCG CAA CG-3' (position 127-147) and downstream primer- 5'-ACG GAT GCA GTT CCT TTC TG-3' (position 707-688) and inner set primers consists of upstream primer-5'-TCT TCC CAG ACG TGG ATT TC-3' (position 152-171) and downstream primer-5'-CTC GAC AAT ACG CTG CTT GA-3'(position 682-663). nPCR was optimized in a 50 μL reaction by the method of Grigg *et al*. (2001) with 10 μL of DNA template of the clinical specimens in Perkin-Elmer automatic thermal cycler (Applied Biosystems, model 2400; Cetus, Norwalk, USA). Amplified product of 579 bp product at the end of first round of amplification and/or 530 bp product at the end of second round of amplification were considered PCR positive.

Table 1a: Primer sequence of nPCR targeting the 3' end of SAG2 gene

Primer	Nucleotide sequence
Outer primer	5' GACCTCGAACAGGAACAC 3'
	5' GACCTCGAACAGGAACAC 3'
Inner primer	5' GAAATGTTTCAGGTTGCTGC 3'
	5' GCAAGAGCGAACTTGAACAC 3'

Table 1b: Primer sequence of nPCR targeting the 5' end of SAG2 gene

Primer	Nucleotide sequence
Outer primer	5' TCTGTTCTCCGAAGTGACTCC 3'
	5' TCAAAGCGTGCATTATCGC 3'
Inner primer	5' ATTCTCATGCCTCCGCTTC 3'
	5' AACGTTTCACGAAGGCACAC 3'

nPCR amplification of SAG2 gene: Two nPCRs, one targeting the 3' end of the SAG2 gene and the other targeting the 5' end of SAG 2 gene were optimized as per the method of Fuentes *et al*. (2001) The nucleotide sequence of the outer and the inner set of primers targeting the 3'end and the 5'end of SAG2 gene are given in Table 1a and 1b respectively. Both the nPCRS were optimized in a 50 µL reaction by the method of Fuentes *et al*. (2001) with 10 µL of DNA template of the clinical specimens in Perkin-Elmer automatic thermal cycler (Applied Biosystems, model 2400; Cetus, Norwalk, USA). For the nPCR targeting the 3' end of SAG2 gene, 300bp product at the end of first round of amplification and/or 221 bp product at the end of second round of amplification were considered PCR positive. For the nPCR targeting the 5' end of SAG2 gene, 340 bp product at the end of first round of amplification and/or 241 bp product at the end of second round of amplification were considered PCR positive.

RESULTS

All the 4 nPCRs were specific to detect only *T. gondii* DNA and the sensitivity was 10 pg of *T. gondii* DNA for B1 gene (primer set 1), 43 pg for B1 gene (primer set 2) and 20ag for both 3' end and 5' end of SAG2 gene respectively.

nPCR targeting B1 gene (primer set 1) was positive in 6 AH, B1 gene (primer set 2) in 2 and both 3'end of SAG2 and 5' end of SAG2 in 4 each. All the four nPCRs were negative in the 12 AH from the controls. The nPCR results of the 12 AH of the patients are given in Table 2. The sensitivity of nPCR targeting B1 gene (primer set 1) is statistically significant compared to other three nPCRs (Chi square test-Yates correction: p = 0.028). Gel Photograph showing the results of nPCR targeting B1 gene (primer set 1 and 2), 3' end and 5' end of SAG2 gene is given in Fig. 1-4 respectively.

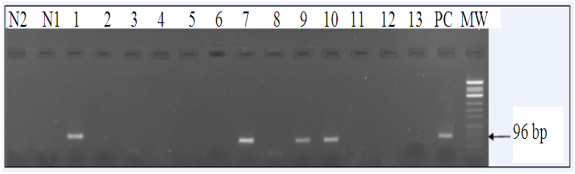

Fig. 1: Agarose gel electrophoretogram of the II round of nPCR using nested primers targeting the B1gene (primer set 1) of *T. gondii* genome on Aqueous Humor (AH) from 13 TRC patients. N2-Negative control II round, N1-negative control I round, (Lane 1, 7, 9 and 10) AH positive for *T. gondii* DNA; (Lane 2-6, 8, 11-13) Negative for *T. gondii* DNA; MW: Molecular Weight marker φ *X* DNA Hinf I digest

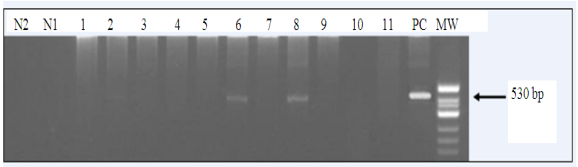

Fig. 2: Agarose gel electrophoretogram of the II round of nPCR using nested primers targeting the B1gene (primer set 2) of *T. gondii* genome on Aqueous Humor (AH) from 11 TRC patients. N2-Negative control II round, N1-negative control I round, (Lane 6 and 8) AH positive for *T. gondii* DNA; Lane 1-6, 7, 9-11-negative for *T. gondii* DNA; MW: Molecular Weight marker: Phi X Hinf I digest

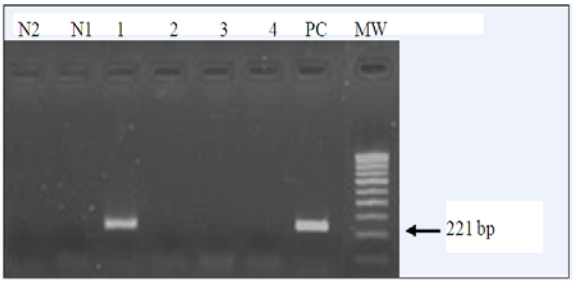

Fig. 3: Agarose gel electrophoretogram of the II round of nPCR using nested primers targeting the 3' end of SAG2 gene of *T. gondii* genome on Aqueous Humor (AH) from 4 TRC patients. N2-Negative control II round, N1-negative control I round, (Lane 1) AH positive for *T. gondii* DNA; (Lane 2-4) negative for *T. gondii* DNA; MW: Molecular Weight marker φ X DNA Hinf I digest

Table 2: Results of nPCR targeting B1 gene (primer set 1 and 2) and SAG2 gene on AH from 12 HIV positive, clinically suspected *Toxoplasma* retinochoroiditis patients

			Results of nPCR*				
Patient number	Age	Sex	B1 gene (primer set 1)	B1 gene (primer set 2)	3'- SAG2 gene	5'- SAG2 gene	Positive by one or more PCR
1	49	M	P	N	N	N	P
2	38	M	P	N	N	N	P
3	25	F	N	N	N	N	N
4	34	M	N	N	P	P	P
5	48	M	P	N	N	N	P
6	34	M	N	N	N	N	N
7	38	M	P	P	P	P	P
8	37	M	N	N	P	P	P
9	33	M	P	N	N	N	P
10	35	M	P	P	P	P	P
11	32	M	N	N	N	N	N
12	45	M	N	N	N	N	N
No. of samples positive by nPCR			6	2	4	4	8

*: The sensitivity of nPCR targeting B1 gene (primer set 1) is statistically significant compared to other nPCR (Chi square test-Yates correction: p = 0.028

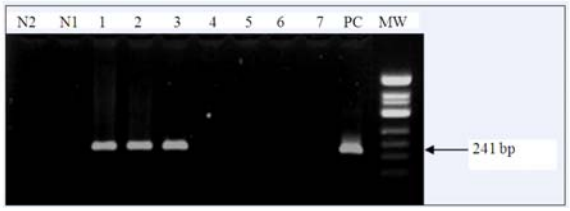

Fig. 4: Agarose gel electrophoretogram of the II round of nPCR using nested primers targeting the B1gene (primer set 1) of *T. gondii* genome on Aqueous Humor (AH) from 13 TRC patients. N2-Negative control II round, N1-negative control I round, (Lane 1, 7, 9 and10) AH positive for *T. gondii* DNA; (Lane 2-6, 8, 11-13) negative for *T. gondii* DNA; MW: Molecular Weight marker: 100 bp ladder

DISCUSSION

Currently the clinical diagnosis of ocular toxoplasmosis is based on the observation of a typical necrotizing lesion on the fundus, response to treatment and serological diagnosis (Jones *et al.,* 2000; Garweg *et al.,* 1996). In HIV positive patients, differentiation of *Toxoplasma* retinochoroiditis, from other disorders is most difficult, as lesions (i) can present with a variety of clinical features, (ii) are not usually associated with retinal scars and (iii) may result in wide spread areas of retinal necrosis that resemble other infections, most commonly CMV retinitis and acute retinal necrosis syndrome (Moorthy *et al.,* 1993; Estrid *et al.,* 2000; Miller *et al.,* 2000). In such cases the aqueous humor analysis is being used as a diagnostic tool for confirmation of TRC (Bou, *et al*., 1999; Miller *et al.,* 2000). Molecular technique-Polymerase Chain Reaction (PCR) for the detection of *T. gondii* genome in ocular fluid blood is a rapid sensitive and reliable diagnostic technique.

B1 gene is a 35 fold repetitive conserved gene within the *T. gondii* genome and was detected among all the strains of *T. gondii* tested till date including several isolates from AIDS patients (Jones *et al.,* 2000; Miller *et al.,* 2000; Brezin *et al.,* 1990). B1 gene was identified by Boothroyd and associates, contains an intron and does not encode a known protein (Brezin *et al.,* 1990) and nPCR targeting B1 gene has been proved to be both specific and sensitive of the primers tested, (Jones *et al.,* 2000; Miller *et al.,* 2000; Brezin *et al.,* 1990; Robert-Gangneux *et al.,* 1999) making it ideal target for PCR amplification (Chan *et al.,* 1994; Manners *et al.,* 1994). The analytical sensitivity of one tachyzoite by nPCR targeting B1 gene in this study is similar to that of other authors (Jones *et al.,* 2000; Moorthy *et al.,* 1993). Surface antigen 2 (SAG2) gene codes for the protein P22 of *T. gondii*, which is a major surface protein known as an attachment ligand that also has good antigenicity and immunogenicity (Fuentes *et al.,* 2001) SAG2 gene is ideally suited for rapid genotyping, as it contains multiple lineage-specific polymorphisms. SAG2 encodes two separate forms of the surface tachyzoite protein p22 that are recognized by strain-specific monoclonal antibodies: type I and III strains share the same protein allele, while type II strains have a second, distinct form (Howe *et al.,* 1997). The usefulness of nPCR targeting SAG2 gene directly on clinical specimens has already been studied (Fuentes *et al.,* 2001; Howe *et al.,* 1997).

All the 4 nPCRs in this study were done on the same DNA and the PCRs were also done almost at the same time, which also rules out degradation of DNA due to storage. The advantage of *in vitro* amplification systems is their high analytical sensitivity. However,

high analytical sensitivity does not necessarily translate into high clinical sensitivity (Persing, 1994). In this study, the 2 nPCRs targeting B1 gene had different analytical and clinical sensitivity. The sensitivity of nPCRs targeting SAG2 gene was higher compared to the B1 gene (Primer set 1), but the clinical sensitivity of B1 gene (primer set 1) was higher than SAG2 gene. The volume of sample tested has a direct bearing on clinical performance if the target copy number is low. The effects of the inhibitors are themselves amplified in a sample containing low copy numbers of the target sequence. Thus, it is important to differentiate analytical sensitivity and clinical sensitivity in the assessment of an amplification technology for clinical use and to realize that an amplification system may not always provide adequate performance despite having high analytical sensitivity (Persing, 1994).

Only the ability to detect *T. gondii* DNA in ocular samples provide direct evidence of the infection as neither culture not the serological tests are useful in immuno compromised individual (Jones *et al.,* 2000). Immuno-deficient patients have markedly higher parasite DNA copy numbers and manifest a feebler immunological degradation of target DNA than immunocompetent patients. Furthermore, the ocular disease in immuno-deficient individuals is an acute form of generalized infection, which is not immunologically controlled. Consequently, the probability of detecting parasite or their DNA in any bodily compartment of immuno deficient patient is much higher than that for immuno competent individuals with a previous or chronic infection (Garweg *et al.,* 2000). In this study, *T. gondii* DNA was detected by one or more of the 4 nPCRs in 66.7% of the 12 patients.

In a study by De-Boer *et al*. (1996), on 7 immunosuppressed patients with TRC, PCR targeting rDNA, was positive in 42.9% (3/7) patients compared to a sensitivity of 50% of B1 gene (primer set 1), 33.3%of SAG2 gene 16% of B1 gene (primer set 2) in this study. Inter-laboratory variability is a confounding factor when analyzing results and trying to compare different methodologies used at different centers (Jones *et al.,* 2000). Predominant of the reports of toxoplasmosis in HIV patients in India, (Shankar *et al.,* 2005;Wadia *et al.,* 2001) Brazil (Colombo *et al.,* 2005; Vidal *et al.,* 2004) and USA(Khan *et al.,* 2005) are on cerebral toxoplasmosis, which is more commoner and life threatening in HIV positive patients. In India, *Toxoplasma* is the most common agent causing focal mass lesions in brain among HIV positive patients (Shankar *et al.,* 2005). A prospective study on a larger scale will throw more light on the prevalence of TRC and *Toxoplasma* encephalitis and the genotype of *T. gondii* among the HIV patients in India.

CONCLUSION

Among the 4 nPCRs, nPCR targeting B1 gene (primer set 1) was the most sensitive, and reliable nucleic acid amplification technique for the laboratory diagnosis of TRC in HIV patients.

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