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Genetic characterization of *Toxoplasma gondii* from autopsy proven cases of AIDS associated cerebral toxoplasmosis in South India

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Abstract

Toxoplasma gondii infection can be devastating in the immunodeficient causing high morbidity and mortality. Due to limited availability of both diagnostic facilities and Highly Active Antiretroviral Therapy (HAART), toxoplasmosis continues to be a significant problem amongst Acquired Immunodeficiency Syndrome (AIDS) patients in India. While scanty literature is available on *T. gondii* isolates in animals in India, little is known about the genetic diversity of the parasite in humans. Therefore, the present study investigated the genetic diversity of *T. gondii* in 25 confirmed cases of cerebral toxoplasmosis developing on the background of human immunodeficiency virus (HIV) infection /AIDS. PCR DNA sequencing was performed at four important genetic loci of *T.gondii*: BTUB, GRA6, alternative SAG2 (alt SAG2) and SAG3 on DNA from tissues obtained at postmortem. The amplified products from all the cases were successfully sequenced except at one locus for one case. Results of the present study suggest that majority of the patients (22/25; 88%) in South India are infected with strains that are recombinants of type II/III and / or strains representing *T. gondii* different from the archetypal lineages I, II, III. In addition, a clonal type III, MAS, MAS variant genotypes were encountered. No clonal type I or II was seen in the present study. In addition, variants were observed at alt SAG2 and SAG3 but BTUB and GRA6 were highly conserved. Single nucleotide polymorphisms were observed mainly at two loci which are coding for surface antigens at alt SAG2 and SAG3. In conclusion, the present study reveals genetic diversity in India amongst strains of *T. gondii* from clinical cases of toxoplasmosis which is in accordance with other recent studies showing a high rate of genetic diversity in this parasite across the globe. There is a need to genotype *T. gondii* from different forms of toxoplasmosis in humans in India.

Keywords: *Toxoplasma gondii;* Human Immunodeficiency Virus; Acquired Immunodeficiency Syndrome; Genotyping; Cerebral toxoplasmosis; Single nucleotide polymorphisms

1. Introduction

Toxoplasma gondii (*T. gondii*) is a ubiquitous protozoan parasite which infects one third of human populations in the world (Dubey, 2008). Most infections in humans are mild, but devastating diseases can occur in congenitally infected children (Wong and Remington, 1994) and in immunocompromised individuals including patients with Acquired Immuno Deficiency Syndrome (AIDS) (Israelski and Remington, 1993; Luft and Remington, 1992). Cerebral toxoplasmosis is one of the common causes of focal brain lesions in patients with AIDS (Luft and Remington, 1992; Ammasari et al., 2000) and is usually the most common cerebral opportunistic disease in both developed and developing countries (Pereira-Chioccola et al., 2009). The incidence of cerebral toxoplasmosis and deaths associated with this condition, in human immunodeficiency virus (HIV) infected individuals has declined considerably with the introduction of Highly Active Anti-Retroviral Therapy (HAART) and primary prophylaxis for toxoplasmosis in the United States of America (Abgrall et al., 2001; Jones et al., 2002). In contrast, in developing countries, cerebral toxoplasmosis still remains an important cause of focal brain lesions in the HIV infected and thus is an important cause of morbidity and mortality (Vidal et al., 2004; Sacktor N., 2002; Alphonso et al., 2009) and in India it is a close second to neurotuberculosis (Satishchandra et al., 2000; Shankar et al., 2005; Shyam Babu et al., 2013).

The seropositivity of *T.gondii* varies in different countries: being less than 30% in USA, Northern Europe, and South East Asia (Allain et al., 1998; Dubey and Jones, 2008),

and greater than 60% in the regions of tropical Africa and Latin America (Fernandes et al., 2009; Pappas et al., 2009). In the Indian subcontinent, the seroprevalence ranges from 5% - 80 % (Sundar et al., 2007; Sucilathangam et al., 2013; Singh et al., 2014); 5.1% - 16.4% in populations of Kyrgyzstan and is 87.4% in Ethiopia (Walle et al., 2013). Toxoplasmic encephalitis might reach up to 40% in patients with AIDS, and 10-30% of them succumb to the parasitic infection (Vidal et al., 2004). Severe toxoplasmosis in immunocompetent human patients is often associated with atypical genotypes (Carme et al., 2002). Type II strains are frequently causing human infection in both congenital as well as in HIV/AIDS patients in North America and Europe (Mercier et al., 2010; Ajzenberg et al., 2002). The isolates of *T. gondii* in human patients and animals in South America are genetically and biologically diverse (Khan et al., 2011). However, there is very scanty information about the epidemiology of *T. gondii* genotypes in humans patients in India particularly in the HIV/AIDS infected.

The genetic diversity of *T. gondii* isolates varies among hosts and geographical regions. In North America and Europe, *T. gondii* has three distinct lineages (type I, II and III) including a fourth clonal lineage referred as type 12 (Khan et al., 2011; Dubey et al., 2011). On the contrary, isolates from South America are genetically and biologically diverse (Dubey et al., 2002, 2007; Lehmann et al., 2006; Rajendran et al., 2012; Carneiro, 2013; Shwab et al., 2014). This was further proven by analyzing isolates from domestic animals in Brazil which revealed 48 genotypes, with four of these isolates being considered to be common clonal lineages, designated as type BrI, BrII, BrIII and BrIV (Pena et al., 2008). Whereas in the African continent (Velmurugan et al., 2008; Al-Kappany et al., 2010), the Middle East and the Arabic peninsula, type II and III strains are very common (Salant et al., 2009; Dubey et

al., 2010). Among the Asian countries, especially in China, a considerable number of cases from animals (Yan et al., 2014; Tian et al., 2014; Li et al., 2015) and humans (Wang et al., 2013; Wang et al., 2015) has revealed predominance of Chinese I (ToxDB#9) genotype apart from archetypal lineages I, II and III (Wang et al., 2013; Wang et al., 2015; Zhou et al., 2009; Tian et al., 2014). As far as India is concerned, limited loci sequencing has been reported in chicken: while one study on SAG2 locus genotyping revealed types II and III (Sreekumar et al., 2003), another recent study on Izatnagar and Chennai isolates revealed type I (Sudan et al., 2015); GRA6 sequencing yielded Type III (Biradar et al., 2014). However, there are no studies on typing of the parasite from human disease.

Therefore, the present study was aimed to characterize *T. gondii* from patients with cerebral toxoplasmosis associated with AIDS registered in a tertiary care neurological center in South India. This was based on studying the DNA sequences at four selective loci [alternative SAG2 (alt SAG2), SAG3, BTUB and GRA6]. We envisage that this would enable development of a protocol for the molecular epidemiological studies, laboratory diagnostics, and control measures including development of candidate vaccines.

2. Materials and methods

2.1 Human brain specimens

A total of twenty five numbers of formalin fixed paraffin embedded tissue blocks of cerebral toxoplasmosis representing the *T. gondii* infection obtained at postmortem from AIDS patients from 2000 to 2014 were included in the study. All patients were immunocompromised, diagnosed as cerebral toxoplasmosis with AIDS. The case definition for toxoplasmosis included the following criteria: (i) progressive neurological deficits; (ii) contrast-enhancing mass lesion(s) on computed tomography and (iii) positive *T. gondii* IgG

serological test on undiluted samples of CSF by Latex Agglutination slide test (Toxogen, Tulip Diagnostics Pvt. Ltd. Goa, India) (iv) histopathological confirmation of *T. gondii* infection. The study was approved by the Scientific Ethics Committee of the Institute (No. NIMHANS/59th IEC/2008 dated 21st march 2008). Written informed consent to utilize the postmortem material for research and teaching was obtained from patients' relatives at the time of autopsy.

2.2 DNA extraction

The diagnosis of cerebral toxoplasmosis was confirmed by histology and immunohistochemistry and the areas with concentrated antigen were marked onto the Formalin Fixed Paraffin Embedded (FFPE) blocks. Total genomic DNA was extracted using Guanidium Thiocyanate method from 100 µm sections from the marked areas of FFPE blocks representative of toxoplasma infection, and aseptically transferred into sterile eppendorf tubes for genetic studies, ensuring use of new blades for each case to prevent carry over contamination (Shi et al., 2002). Briefly, tissue sections were deparaffinised using 1.5ml Xylene (molecular grade) at 56°C overnight. After complete removal of wax, the tissue was digested using 400µl Guanidium Thiocyanate Buffer (20mM Tris, pH 8.0; 1M Guanidium Thiocyanate; 20mM β-mercaptoethanol; 0.5% Sarcosyl) containing Proteinase K (6mg/ml), incubated at 55°C overnight. After complete digestion, Proteinase K was inactivated by incubating the samples at 95°C for 10 minutes. Following this, genomic DNA was extracted by the conventional technique using Phenol:Chloroform (Sambrook and Russell, 2001).

2.3 B1 PCR

Total genomic DNA extracted from the tissue blocks of autopsy proven cerebral toxoplasmosis from all the 25 cases were subjected to nested (n) Polymerase Chain Reaction (PCR) for B1 gene of *T.gondii* using the primers and conditions described earlier (Guy and Joynson, 1995). Cases which were B1 nPCR positive were further subjected to PCR based multilocus sequence analyses. Beta globin gene amplification served as internal control for DNA extraction and amplification from all samples (Saiki et al., 1985).

2.4 Multilocus PCR sequence analyses

Four independent loci of *T.gondii* viz. SAG3, BTUB, GRA6 and alt SAG2 were amplified by a multilocus PCR as described (Su et al., 2006) with minor modification. GT1 (type I), PTG (type II), CTG (type III) served as reference strains (kindly provided by Dr. Su, Associate Professor Department of Microbiology, The University of Tennessee, USA). Briefly, the multiplex PCR reaction was carried out in 25µl of volume containing 1xPCR buffer, 2mM $MgCl_2$, 200µM each of the deoxynucleoside triphosphates (dNTPs), 0.5µM each of the forward and reverse primers, (0.03units/µl) of Q5 high fidelity hot start Taq DNA polymerase (cat. No. M0493S, New England Biolabs) and 1µl of extracted genomic DNA. The reaction mixture was first treated at 98^{o}C for 30 seconds, followed by 35 cycles of 98^{o}C for 10 seconds, 55^{o}C for 30 seconds and 72^{o}C for 30seconds, and final extension at 72^{o}C for 2 minutes. The amplified products were diluted 1:2 in sterile DNAse free water and used for second round of amplification (nPCR) with internal primers for each marker separately. Five µl of PCR products were analyzed by electrophoresis in 1.5 % agarose gel containing 0.3µg/ml ethidium bromide and visualized on a transilluminator. The nested PCR products were purified with ‘‘Wizard SV gel and PCR Clean-up System kit’’ (PROMEGA) according to the manufacturer’s instructions for sequencing by Sangers di-deoxy sequencing method.

2.5 Sequence analysis

The fragments were sequenced with both forward and reverse internal primers for each marker (alt SAG2, SAG3, BTUB and GRA6) to obtain specific forward and reverse sequences. Sequencing was carried out using Big Dye Terminator (BDT) chemistry version 3.1 (ABI 3730XL Genetic Analyzer of Applied Biosystems). Nucleotide sequences were analyzed and manually assembled and aligned for comparison using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) multiple sequence alignment program. The sequences of the GRA6 locus were aligned with Type I - RH (accession no: JN649063.1), Type II - ME49 (accession no: AF239285.1) Type III - NED, CTG, C56 (accession nos: AF239286.1, JX044207.1, DQ512729.1, respectively) sequences. BTUB locus sequences were aligned with Type I - RH, GT1 (accession nos: JX045508.1, JX045509.1 respectively), Type II - Beverly (accession no: AF249702.1) and Type III - CTG, C56 (accession nos: JX045537.1, AF249703.1 respectively) sequences. Alt SAG2 locus sequences were aligned with Type I - RH (accession no: JX045478.1), Type II - Beverly (accession no: AF249697.1) Type III C56, NED (accession nos: AF249698.1, AF357579.1 respectively) sequences. SAG3 locus sequences were aligned with Type I - RH (accession no: AF340227.1), Type II - PTG (accession no: JX218226.1) Type III - CTG (accession no: JX218227.1) sequences. Recombinant sequences that were thus obtained were deposited in GenBank and the accession numbers of the nucleotide sequences are cited in the results section.

3. Results

3.1 Study population and Pathology

Cerebrospinal fluid from all 25 cases collected at autopsy tested positive for antitoxoplasma IgG antibodies by Latex Agglutination test. All 25 cases had multifocal

necrotizing hemorrhagic lesions involving basal ganglia / thalamus, cerebellum, brain stem and smaller lesions along the grey white junction corresponding to the lesions identified on ante mortem CT/MRI scans. Histologically the lesions were necrotizing and hemorrhagic with hypertrophic endarteritic changes of the arterioles. Presence of tachyzoite forms of *T.gondii* was confirmed by immunohistochemistry using tachyzoite specific antibodies recognizing p30 antigen.

3.2 Multilocus PCR Sequencing based Genotypes of T.gondii

Total genomic DNA extracted from all 25 FFPE tissues of autopsy proven cases of cerebral toxoplasmosis were positive for B1 gene of the parasite by nPCR. The isolated genomic DNA was subsequently subjected to multilocus nPCR based amplification at four loci viz. SAG3, alt SAG2, BTUB and GRA6 and the purified amplicons were successfully sequenced except for one sample at alt SAG2 locus (sample ID number A27/01). The sequences were analyzed using CLUSTALW multiple sequence alignment program (Table 1) and aligned with Type I (RH, GT1), Type II (PTG, Beverley, ME49) and Type III (CTG, C56, NED) sequences available in the Gene Bank database. Out of the 25 cases of cerebral toxoplasmosis, *T. gondii* DNA amplified at four genetic loci, 17 genotypes were identified: one of Type III which had 100% homology with the available gene sequences of type III (CTG, C56 and NED); one which showed homology to MAS, one MAS variant, and two were non-archetypal genotypes. Of the 12 recombinant genotypes of types II/III, seven had variation at alt SAG2 and SAG3, which differed from the types known so far (Table 1).

3.3 Recombinant genotypes of II and III

Of these 22 cases (12 types) with recombinants of II/III, seven had higher genetic diversity and also showed variation at one or more of these loci (alt SAG2 and SAG3).

BTUB and GRA6 loci were highly conserved, and showed combinations of type II/III except for one strain which showed type I genotype at locus BTUB (Table 1; sample ID. A26/04) and another case which had a variant of type I at SAG3 locus along with type II variant at alt SAG2 locus, which we classified as non-archetypal genotypes. The sequences of amplified products of alt SAG2 and SAG3 loci appeared to be recombinants of genotypes II or III: Single Nucleotide Polymorphisms (SNPs) were noted in nine samples – in which six samples showed genetic variation in alt SAG2 alone, one sample showed genetic variation in SAG3 alone and two others showed genetic variation at both loci - alt SAG2 and SAG3 (Table 1). A total of four variants of alt SAG2 were recognized (Accession nos. KT248834 to KT248837; Fig.1): first type variant was found in sample nos. A22/14, A5/06, A23/07, and A89/02 wherein SNPs were seen at positions 227A>T; 283G>T; 312A>G; 383T>C; and A>C at 423 (numbered as per Genbank accession no: AF249697.1). The second variant of type II was seen in three samples M3/11, A12/10, A69/03 which had T>G at position 212 and A>C at 423; the third variant was seen in sample no. A32/07 which had T/T at position 212 and A>C at 423 position, whereas alt SAG2 locus in sample no. A28/09 did not have any SNP, but appeared to be a recombinant of types II/III. SAG3 locus had two variants of type III and one variant of type I (Accession nos. KT 310096 to KT 310098; Fig. 2): sample no. A60/01 had two SNPs – Y at 63bp and a C>T at 120; A12/10 also had two SNPs one at 88bp – GG>TA and at 100bp, a C>T (numbered as per Genbank accession no: JX218227.1). Also, sample no. A23/07 had two variations of type I viz. A>R at 87, A>C and G>C at 135 and 143 positions (numbered as per Genbank accession no: AF340227.1) respectively.

4. Discussion

Cerebral toxoplasmosis is one of the major causes of CNS mass lesions in the brain in HIV/AIDS patients both in India (Shankar et al., 2005) and the world (Luft and Chua, 2000; Gaspar et al., 2002; Manzardo et al., 2005). Moreover, HAART therapy is not widely available and toxoplasmosis continues to be a significant problem amongst AIDS patients in India (Shankar et al., 2005). The high prevalence rate of toxoplasmosis in some parts of the world such as South America and Africa, coupled with a greater severity of clinical manifestations in developing nations and limitations of currently available antiparasite drug treatments warrant an urgent need for alternative therapeutic strategies (Jongert et al., 2009). The discovery of atypical strains in the South American region which are more virulent than the classic European types I, II and III advocate next generation research efforts towards development of a polyvalent vaccine against *T. gondii* encompassing all the genotypes prevailing in the world (Jongert et al., 2009). Towards such a broad goal, we undertook this study to characterize genetic variation in *T. gondii* from cases of human cerebral toxoplasmosis in India. This is particularly relevant since, there are no studies on genetic characterization of human toxoplasmosis from India.

As evidenced by various studies, genetic variation in *T. gondii* strains is strongly linked to the geographical origin of infection. Initial studies revealed that *T. gondii* isolates from human patients from North America and Europe fell into three distinct lineages of type I, II and III with a predominance of type II strains (Darde et al., 1987; Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Howe et al., 1997). This was subsequently confirmed on a larger cohort wherein European patients had infection by type II, and non-type II isolates were mainly recorded in patients who acquired toxoplasmosis outside Europe along with type

I, II, III clonal lineages (Ajzenberg et al., 2009). Also in support is a recent study on congenital toxoplasmosis from Romania (Eastern Europe) which revealed type II genotype based on the microsatellite genotyping analysis (Costache et al., 2013). In addition, a study from Germany also identified type II in a majority of the patients suffering from toxoplasmosis regardless of the clinical manifestation of infection (Herrmann et al., 2014). A fourth clonal lineage termed as haplogroup 12 has also been recorded (Khan et al., 2011; Dubey et al., 2011). However, later studies revealed that the parasite has a highly unusual population structure and the distribution of genotypes varied geographically: studies from Brazil, tropical regions of South America indicated *T. gondii* strains isolated from humans and animals, to be biologically and genetically different from those found in North America and Europe (Pena et al., 2008). Isolates of *T. gondii* from animals in South America showed higher genetic diversity (Dubey et al., 2002, 2007; Lehmann et al., 2006; Khan et al., 2007; Pena et al., 2008; Rajendran et al., 2012; Pena et al., 2013; Shwab et al., 2014). Interestingly, genotyping of Brazilian cases of toxoplasmosis with different clinical manifestations including patients with HIV/AIDS, revealed predominantly a new genotype ToxoDB#65 by PCR-RFLP (Ferreira et al., 2011). These strains had previously been identified from farm / pet animal infections in Brazil, indicating the importance of animals as the reservoir of human infection (Silva et al., 2014). Yet another study from Brazil using RFLP at 11 loci revealed a new genotype BrII in 29% of cases of congenital toxoplasmosis (Carneiro et al., 2013). Together, these studies indicated that genetic variation in *T. gondii* strains is strongly linked to the geographical origin of infection. Various studies from Brazil (Darde´, 2004; Ferreira et al., 2006; Ferreira et al., 2011; Dubey et al., 2004, 2005, 2007; Gallego et al., 2006; Peyron et al., 2006; Belfort-Neto et al., 2007), showed that for genotyping non-clonal strains, markers designed to discriminate the North American and European isolates may be

inappropriate. Also, a recent study revealed that strains previously defined as atypical by unusual RFLP patterns, yielded both new variants of an existing lineage and completely new lineages after re-evaluation by DNA sequencing (Khan et al., 2011).

In sub-Saharan Africa, non-archetypal genotypes named Africa 1, 2, and 3 have been isolated from humans and domestic animals in addition to the three major lineages (Ajzenberg et al., 2009; Bontell et al., 2009; Mercier et al., 2010). In North Africa, the Middle East and the Arabic peninsula, type II and III strains were found to be very common (Velmurugan et al., 2008; Al-Kappany et al., 2010; Dubey et al., 2010; Salant et al., 2009). Whereas another study on congenital human toxoplasmosis from north Africa identified recombinant genotypes majorly of I/III and also of I/II using direct sequencing at six loci viz. 3'SAG2, 5'SAG2, SAG3, BTUB, GRA6, and APICO. (Boughattas et al., 2010). Also, an unusual combination of type I/III alleles was revealed in an HIV infected African patient with severe encephalitis (Genot et al., 2007).

Amongst the Asian countries, especially in China, a considerable number of cases from animals (Chao Yan et al., 2014; Tian et al., 2014; Li et al., 2015) and humans (Wang et al., 2013; Lin Wang et al., 2015) revealed the predominance of Chinese I (ToxDB#9) genotype apart from archetypal lineages type II (Tian et al., 2014). In the present study, human brain specimens from 25 fatal cases of CNS toxoplasmosis in AIDS patients, were subjected to characterization by multilocus PCR DNA sequencing at four genetic markers (alt.SAG2, SAG3, BTUB, GRA6) and sequenced bi-directionally, which revealed that only one sample harbored type III, and two were akin to MAS. High genetic diversity was seen amongst the strains. We could not sequence one sample at alt SAG2 locus since repeated attempts failed to amplify it. Such a failure to amplify some of the single copy genes of the

parasite has been recognized earlier (Khan et al., 2005). Although the present study was on a limited number of samples and analyzed at limited loci, it was interesting to note that it revealed 17 genotypes with majority being recombinants of type II/III except only one specimen, which harbored type I allele at BTUB locus Some isolates also showed many SNPs at two loci i.e., alt.SAG2 and SAG3 as compared to clonal types II and III (alt.SAG2-Beverly-clonal type II and PTG-clonal type III). Limited genotyping on chicken isolates in India, showed type II and type III genotypes based on SAG2 genotyping (Sreekumar et al., 2003), type I genotype based on the sequence analysis of SAG2 (Singh et al., 2011), SAG3 (Sudan et al., 2012) and type III genotypes based on GRA6 (Biradar et al., 2014). Thus, our present results on genotypes of *T.gondii* from human cases of cerebral toxoplasmosis cannot be compared with the genotypes from chicken since the latter was a very limited study.

Overall our results indicate that in South India, genetic diversity was observed in *T.gondii* with majority of them being recombinant genotypes of types II/III and/or strains representing *T. gondii* different from the archetypal lineages I, II, III. In addition, some of them had SNPs mainly at two loci viz. alt SAG2 and SAG3. Since majority of these variants had SNPs and similar SNPs were noted amongst the cases studied, they could become new markers for further investigation. The SNPs seen in the present study were confined to the antigen coding loci, which might have resulted due to the diversifying selection on these genes (Lehmann et al., 2000) and may be relevant keeping in mind the development of vaccines and diagnostics.

Conclusion

The present study indicates that majority of *T.gondii* causing CNS toxoplasmosis in India are either recombinant genotypes of types II/III, and / or strains representing *T.gondii* different from the archetypal lineages I, II, III. Also variants were mainly noted at two genetic loci SAG3 and alt SAG2, but BTUB and GRA6 were highly conserved, and support other recent studies showing genetic variation in *T. gondii*. However, future studies with more extensive sequencing at more loci including microsatellites and other house-keeping genes and on a larger sample size involving different regions of India may be required to further characterize the extent of molecular diversity of *T. gondii* in the country. These results may have important implications for diagnostic protocols, drug treatments and development of vaccines.

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Conflict of interest

The authors declare no conflict of interest.

Figure legend

Fig. 1. Alt SAG2 variants in CNS toxoplasmosis

Fig. 2. SAG3 variants in CNS toxoplasmosis

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XI04_A23-07_SAG2[Toxoplasma       TTGTGTTCAAGTTCGCTCTTGCGTCCTCCACCGAGACGCCAGCGCCCATT 207
XI04_A89-02_SAG2[Toxoplasma       TTGTGTTCAAGTTCGCTCTTGCGTCCTCCACCGAGACGCCAGCGCCCATT 204
XII104_A5-06_SAG2[Toxoplasma      TTGTGTTCAAGTTCGCTCTTGCGTCCTCCACCGAGACGCCAGCGCCCATT 216
XII104_A22-14_SAG2[Toxoplasma     TTGTGTTCAAGTTCGCTCTTGCGTCCTCCACCGAGACGCCAGCGCCCATT 216
IX160_A69_03_SAG2[Toxoplasma      TTGTGTTCAAGGTCGCTCTTGCGTCCACCACCGAGACGCCAGCGCCCATT 249
IX160_M3_11_SAG2[Toxoplasma       TTGTGTTCAAGGTCGCTCTTGCGTCCACCACCGAGACGCCAGCGCCCATT 216
IX-18_A12-10_SAG2                 TTGTGTTCAAGGTCGCTCTTGCGTCCACCACCGAGACGCCAGCGCCCATT 210
XI74_A28-09_SAG2[Toxoplasma       TTGTGTTCAAGTTCGCTCTTGCGTCCACCACCGAGACGCCAGCGCCCATT 210
IX160_A32_07_SAG2[Toxoplasma      TTGTGTTCAAGTTCGCTCTTGCGTCCACCACCGAGACGCCAGCGCCCATT 246
SAG2T2BEVERLY                     TTGTGTTCAAGTTCGCTCTTGCGTCCACCACCGAGACGCCAGCGCCCATT 250
                                  *********** ************** ***********************

XI04_A23-07_SAG2[Toxoplasma       GAGTGCACTGCCGGCGCAACGAAGACTGTTGATGCACCCTCCAGTGGTTC 257
XI04_A89-02_SAG2[Toxoplasma       GAGTGCACTGCCGGCGCAACGAAGACTGTTGATGCACCCTCCAGTGGTTC 254
XII104_A5-06_SAG2[Toxoplasma      GAGTGCACTGCCGGCGCAACGAAGACTGTTGATGCACCCTCCAGTGGTTC 266
XII104_A22-14_SAG2[Toxoplasma     GAGTGCACTGCCGGCGCAACGAAGACTGTTGATGCACCCTCCAGTGGTTC 266
IX160_A69_03_SAG2[Toxoplasma      GAGTGCACTGCCGGCGCAACGAAGACTGTTGAGGCACCCTCCAGTGGTTC 299
IX160_M3_11_SAG2[Toxoplasma       GAGTGCACTGCCGGCGCAACGAAGACTGTTGAGGCACCCTCCAGTGGTTC 266
IX-18_A12-10_SAG2                 GAGTGCACTGCCGGCGCAACGAAGACTGTTGAGGCACCCTCCAGTGGTTC 260
XI74_A28-09_SAG2[Toxoplasma       GAGTGCACTGCCGGCGCAACGAAGACTGTTGATGCACCCTCCAGTGGTTC 260
IX160_A32_07_SAG2[Toxoplasma      GAGTGCACTGCCGGCGCAACGAAGACTGTTGAGGCACCCTCCAGTGGTTC 296
SAG2T2BEVERLY                     GAGTGCACTGCCGGCGCAACGAAGACTGTTGAGGCACCCTCCAGTGGTTC 300
                                  ******************************** *****************

XI04_A23-07_SAG2[Toxoplasma       CGTTGTCTTCCGATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 307
XI04_A89-02_SAG2[Toxoplasma       CGTTGTCTTCCGATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 304
XII104_A5-06_SAG2[Toxoplasma      CGTTGTCTTCCGATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 316
XII104_A22-14_SAG2[Toxoplasma     CGTTGTCTTCCGATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 316
IX160_A69_03_SAG2[Toxoplasma      CGTTGTCTTCCAATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 349
IX160_M3_11_SAG2[Toxoplasma       CGTTGTCTTCCAATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 316
IX-18_A12-10_SAG2                 CGTTGTCTTCCAATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 310
XI74_A28-09_SAG2[Toxoplasma       CGTTGTCTTCCAATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 310
IX160_A32_07_SAG2[Toxoplasma      CGTTGTCTTCCAATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 346
SAG2T2BEVERLY                     CGTTGTCTTCCAATGTGGGGATAAACTAACCATCAGTCCCAGTGGCGAAG 350
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XI04_A23-07_SAG2[Toxoplasma       GTGATGTCTTTTATGGCAAGGAATGCACAGACCCGAGGAAGTTGACGACT 357
XI04_A89-02_SAG2[Toxoplasma       GTGATGTCTTTTATGGCAAGGAATGCACAGACCCGAGGAAGTTGACGACT 354
XII104_A5-06_SAG2[Toxoplasma      GTGATGTCTTTTATGGCAAGGAATGCACAGACCCGAGGAAGTTGACGACT 366
XII104_A22-14_SAG2[Toxoplasma     GTGATGTCTTTTATGGCAAGGAATGCACAGACCCGAGGAAGTTGACGACT 366
IX160_A69_03_SAG2[Toxoplasma      GTGATGTCTTTTATGGCAAGGAATGCACAGACTCGAGGAAGTTGACGACT 399
IX160_M3_11_SAG2[Toxoplasma       GTGATGTCTTTTATGGCAAGGAATGCACAGACTCGAGGAAGTTGACGACT 366
IX-18_A12-10_SAG2                 GTGATGTCTTTTATGGCAAGGAATGCACAGACTCGAGGAAGTTGACGACT 360
XI74_A28-09_SAG2[Toxoplasma       GTGATGTCTTTTATGGCAAGGAATGCACAGACTCGAGGAAGTTGACGACT 360
IX160_A32_07_SAG2[Toxoplasma      GTGATGTCTTTTATGGCAAGGAATGCACAGACTCGAGGAAGTTGACGACT 396
SAG2T2BEVERLY                     GTGATGTCTTTTATGGCAAGGAATGCACAGACTCGAGGAAGTTGACGACT 400
                                  ******************************** *****************

XI04_A23-07_SAG2[Toxoplasma       GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 407
XI04_A89-02_SAG2[Toxoplasma       GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 404
XII104_A5-06_SAG2[Toxoplasma      GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 416
XII104_A22-14_SAG2[Toxoplasma     GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 416
IX160_A69_03_SAG2[Toxoplasma      GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 449
IX160_M3_11_SAG2[Toxoplasma       GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 416
IX-18_A12-10_SAG2                 GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 410
XI74_A28-09_SAG2[Toxoplasma       GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 410
IX160_A32_07_SAG2[Toxoplasma      GTCCTTCCAGGTGCGGTCTTGACAGCTAAGGTCGAGCAGCCCCCGAAAGG 446
SAG2T2BEVERLY                     GTCCTTCCAGGTGCGGTCTTGAAAGCTAAGGTCGAGCAGCCCCCGAAAGG 450
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Fig. 1. Alt SAG2 variants in CNS toxoplasmosis

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SAG3T2PTG                       CAAAGTGAGAGTTGCTGGGAACCCCAGAAAGTGGGAGAGGGGCGGAGGCG 98
VII193_A23-07_SAG3F_G06.ab1     CAAAGTGAGAGTTGCCGGGAACCCCAGAAAGTGGGGGAGRGGCGGAGGCG 68
SAG3T1RH                        CAAAGTGAGAGTTGCCGGGAACCCCAGAAAGTGGGGGAGAGGCGGAGGCG 98
SAG3T3CTG                       CAAAGTGAGAGTTGCCGGGAACCCCAGAAAGTGGGGGAGAGGCGGAGGCG 98
VII193_A12-10_SAG3R_D06.ab1     CAAAGTGAGAGTTGCCGGGAACCCCAGAAAGTGGGGGAGATACGGAGGCG 99
XII31_A60-01_SAG3REV_F10.ab1    CAAAGTGAGAGTTGCYGGGAACCCCAGAAAGTGGGGGAGAGGCGGAGGCG 100
                                *************** ******************* ***   ********

SAG3T2PTG                       GCCATCCAGGAAGCGGAGGATTGCAGCCAGGAACTGACGGGGAAACTCAA 148
VII193_A23-07_SAG3F_G06.ab1     GCCATCCAGGAAGCGGAGGATTGCAGCCGGGAACTGACGGGGAAACTCAA 118
SAG3T1RH                        GCCATCCAGGAAGCGGAGGATTGCAGCCGGGAACTGAAGGGGAAAGTCAA 148
SAG3T3CTG                       GCCATCCAGGAAGCGGAGGATCGCAGCCGGAAACTGACGGGGAAACTCAA 148
VII193_A12-10_SAG3R_D06.ab1     GCTATCCAGGAAGCGGAGGATCGCAGCCGGAAACTGACGGGGAAACTCAA 149
XII31_A60-01_SAG3REV_F10.ab1    GCCATCCAGGAAGCGGAGGATTGCAGCCGGAAACTGACGGGGAAACTCAA 150
                                ** ****************** ****** * ****** ******* ****

SAG3T2PTG                       GCTGGAACAGGAAGTTCAGCCGGCGCGAGTTCGCGAATGGCTTCCGTTGC 198
VII193_A23-07_SAG3F_G06.ab1     GCTGGAACAGAAAGTTCAGCCGGCGCGAGTTCGCGAATGGCTTCCGTTGC 168
SAG3T1RH                        GCTGGAACAGAAAGTTCAGCCGGCGCGAGTTCGCGAATGGCTTCCGTTGC 198
SAG3T3CTG                       GCTGGAACAGAAAGTTCAGCCGGCGCGAGTTCGCGAATGGCTTCCGTTGC 198
VII193_A12-10_SAG3R_D06.ab1     GCTGGAACGGAAAGT-CAGCCGGCGCGAGT-CGCGAATG-------CT-- 188
XII31_A60-01_SAG3REV_F10.ab1    GCTGGAACAGAAAGT-CAGCCGGCGCGAGT-CGCGAATG-------CTAC 191
                                ******** * **** ************** ********       *
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Fig. 2. SAG3 variants in CNS toxoplasmosis

Table 1: Multilocus PCR Sequencing of T.gondii from cases of CNS toxoplasmosis Details of variation observed at four loci by PCR Sequencing of *T.gondii* from cases of CNS toxoplasmosis

			Summary of multilocus PCR Sequencing				
S.No.	**Samples**	**Age/ Sex**	**BTUB**	**GRA6**	**Alt SAG2**	**SAG3**	**Genotype**
1	A10/08	54/M	III	III	III	III	III
2	A75/02	40/M	III	III	II	III	MAS
3	A60/01	30/M	III	III	II	IIIv	MAS (Variant)
4	A5/01	38/M	III	III	II	II	Recombinant
5	A57/01	45/F	III	III	II	II	Recombinant
6	A84/00	42/M	III	III	II	II	Recombinant
7	A89/01	32/F	III	III	II	II	Recombinant
8	A32/07	47/M	III	III	IIv	II	Recombinant + Variant
9	M3/11	50/M	III	III	IIv	II	Recombinant +Variant
10	A22/14	40/M	III	III	IIv	II	Recombinant +Variant
11	A5/06	32/M	III	III	IIv	II	Recombinant +Variant
12	A39/06	33/M	III	III	III	II	Recombinant
13	A1/10	44/M	III	II	II	III	Recombinant
14	A12/10	35/F	III	II	IIv	IIIv	Recombinant +Variant
15	A23/07	45/M	III	II	IIv	Iv	Non-Archetype
16	A28/09	48/M	III	II	II or III	II	Recombinant
17	A13/02	30/F	II	III	II	II	Recombinant

18	A15/00	20/M	II	III	II	II	Recombinant
19	A23/08	51/M	II	III	II	II	Recombinant
20	A84/02	40/M	II	III	II	II	Recombinant
21	A89/02	25/M	II	III	IIv	II	Recombinant +Variant
22	A69/03	32/F	II	III	IIv	II	Recombinant +Variant
23	A24/07	40/M	II	III	III	II	Recombinant
24	A26/04	50/M	I	III	III	II	Non-Archetype
25	A27/01	32/M	II	III	Not done	II	Recombinant

v: details of variants are provided in figures 1 and 2

Genetic characterization of *Toxoplasma gondii* from autopsy proven cases of AIDS associated cerebral toxoplasmosis in South India

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Highlights:

- *T.gondii* was genotyped by sequencing at 4 loci in 25 cases of cerebral toxoplasmosis with AIDS
- 17 genotypes were observed.
- 12 recombinants of II/III; 1 each of types III; MAS; MAS variant & 2 non-archetypes were observed
- Alt SAG2 & SAG3 had high genetic diversity; BTUB & GRA6 loci were highly conserved
- Single Nucleotide Polymorphisms were detected at two loci: alt SAG2 and SAG3