Genotyping of *Toxoplasma gondii* in Cerebral and Ocular Toxoplasmosis

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ABSTRACT

*Toxoplasma gondii* is an obligate intracellular protozoon, spread world-wide and capable of infecting birds and mammals. Genetic information on *T. gondii* type that causes human toxoplasmosis is limited. In this study, genetic analysis of SAG2 locus was used to determine the genotype of *T. gondii* from cases with cerebral and ocular toxoplasmosis in Indonesia. Genotype determination was done directly on the clinical samples. A number of 28 cerebrospinal fluid and 8 vitreous humor positively infected with *T. gondii* underwent PCR-RFLP to classify each isolate into one of three genotypes of *T. gondii*. Type I was the most common found suggesting that cerebral and ocular toxoplasmosis in Indonesia is mostly caused by type I strain of *T. gondii*.

Keywords: *Toxoplasma gondii*, genotype, PCR-RFLP, cerebrospinal fluid, ocular fluid

Introduction

*Toxoplasma gondii* is an obligate intracellular protozoan of phylum Apicomplexa infecting birds and mammals. It is estimated that almost one-third of global population is infected with *T. gondii* [1, 2]. Clinical manifestation of toxoplasmosis varies and influenced by several factors, such as duration of exposure, geographic, parasite genetics, immune status of the host, and time of infection [3]. In immunocompetent individuals, *Toxoplasma* infection only shows minor symptoms or even asymptomatic. On the other hand, if the infection occurs in immunodeficient individuals, clinical manifestation might be fatal or even lethal [2, 4].

Information on dynamics of toxoplasmosis cases, either continuously or periodically, is still limited and very few study reports on *T. gondii* genotype infecting human, hence *T. gondii* type is related to its pathogenic and biological character which is considered to play role in the pathogenesis and management of toxoplasmosis [5].

*Toxoplasma* encephalitis (TE) is one of the most common neurological infection in AIDS patients. It causes significant morbidity and mortality [6]. Besides TE, *T. gondii* is one of the most common cause of retinochoroiditis both in immunocompetent and immunocompromised individuals [7]. Variation in biological characteristics of each *T. gondii* type is considered to cause different clinical manifestations in human.

Virulence of *T. gondii* in animals varies, depending on the type [5]. More than 95% type of *T. gondii* isolated from North America and Europe belongs to one of three lineages, which referred to type I-III (Howe and Sibley, 1995). Genetically, the difference amongst each type is only 1 – 2%, but virulence differs significantly [2].

Determination of three lineages by Howe and Sibley in 1995 was based on analysis of six single-copy independent locus, one of which was *surface antigen 2* (SAG 2) which is abundantly present on the parasite surface [8]. Other single gen target to determine *T. gondii* genotype is GRA 6 [9]. This study reports the type of *T. gondii* among Indonesian patients with cerebral and ocular toxoplasmosis.

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Material and Methods

Study sample

Samples consisted of 88 cerebrospinal fluid from AIDS patients with cerebral disorder, as part of neuroaids study. While the 64 vitreous humor, obtained from patients clinically diagnosed as atypical uveitis and has been screened for Toxoplasma infection. All samples were stored in -30°C in Parasitology Laboratory, Faculty of Medicine, Universitas Indonesia. The study was approved by the ethical committee of Faculty of Medicine, Universitas Indonesia number 721/UN2.F1/ETIK/2016.

DNA isolation

DNA extraction was done by boiling the samples for 10 minutes at 100°C [11]. For cerebral fluid, it was preceded by centrifugation of 1 mL of samples for 10 minutes at 10,000 rpm and leaving 150 µL sediment for boiling. For vitreous humor, samples were directly boiled due to very limited volume of samples obtained (≤ 100 µL).

Detection of T. gondii by PCR

To confirm T. gondii infection in cerebrospinal fluid and vitreous humor, nested PCR were performed targeting B1 gene, following the procedure by Alfonso et al. (2008) [11].

Target DNA amplification for genotyping

Strain type of T. gondii was determined by PCR-Restriction Fragment Length Polymorphism (RFLP) of SAG2 gene with PCR cycle condition in accordance to the protocol used by Fuentes et al. (2001), Howe et al. (1997) [4, 10]. The PCR was optimized and run in 20 μL reaction using TopTaq Polymerase Master Mix Kit (Qiagen, GmbH, cat no. 200403) and 2 μL DNA template on MJ Research PTC 200 thermocycler. The primers used are presented in the Table 1. Negative control (pure water) and positive control (T. gondii RH strain, generous gift from Indonesian Research Centre for Veterinary Science) were always included in every PCR.

Optimum nested PCR condition for 5’-SAG2

Table 1. Primer sequences used [4, 12]

| Target genes | Forward | Reverse | Amplicons |
|--------------|---------|---------|-----------|
| 5’-SAG2      | nest 1(SAG2.F4): GCTACCTCGAACAGGAACAC | nest 1(SAG2.R4): GCATCAACAGTCTTCGTTGC | 241 bp |
|              | nest 2 (SAG2.F): GAAATGTTCAGGTGCTGC | nest 2 (SAG2.R2): GCAAGAGCGAACTTGACAC |
| 3’-SAG2      | nest 1 (SAG2.F3): TCTGTTCTCGGAAGTAGCTCC | nest 1 (SAG2.R3): TCAAAGCGTGCA TTATCGC | 221 bp |
|              | nest 2 (SAG2.F2): ATTCTCATGCTCGGCTTC | nest 2 (SAG2.R): AACGTTTCAGGAAGG CACAC |
Table 2. PCR condition for 5’-SAG2 and 3’-SAG2

| No | Condition          | 5’-SAG2       | 3’-SAG2       |
|----|--------------------|---------------|---------------|
| 1  | Denaturation       | Nested I & II: 94°C, 5 minutes | Nested I & II: 94°C, 5 minutes |
| 2  | Annealing          | nested I: 60°C, 45 seconds nested II: 58°C, 45 seconds | nested I: 58°C, 45 seconds; nested II: 55°C, 45 seconds |
| 3  | Elongation         | Nested I & II: 72°C, 1 minutes | Nested I & II: 72°C, 1 minutes |
| 4  | Cycle number       | 40            | 40            |

and 3’-SAG2 genes is presented in Table 2. The last cycle was extended for 10 minutes at 72°C. The 241 bp and 221 bp of 5’-SAG2 and 3’-SAG2 respectively PCR products were visualized on 2% agarose gel electrophoresis.

The PCR products of 3’-SAG2 was digested with HhaI restriction enzymes by 4 hours incubation at 37°C, followed by inactivation at 65°C for 20 minutes while 5’-SAG2 PCR product was digested with Sau3AI, incubated at 37°C for 3 hours and inactivated at 80°C for 20 minutes. The reaction was performed in 15 µL volume. The result was analyzed by electrophoresis on 2% agarose gel with TBE buffer solution and visualized by exposure to UV light. GeneRuler 50-bp DNA Ladder (Thermo Scientific) was used as molecular size marker. Interpretation of the result to identify the T. gondii type I/II/III.

Results and Discussion

The PCR examination of B1 gene revealed 28 out of 88 (31.82%) cerebrospinal fluid samples and 8 out of 64 (12.5%) ocular fluid were positive for T. gondii infection. PCR of the 28 spinal fluid previously B1 gene positive, showed fewer positive result which were 26/28 and 4/28 samples respectively on 5’-SAG2 and 3’-SAG2. Similar result was obtained for the 8 ocular fluid samples, which were 100% positive on the 5’-SAG2 PCR however only 2/8 samples were positive on 3’-SAG2 gene.

RFLP of gene SAG2 PCR products

The size of amplicon for 5’-end was 241 bp and for 3’-end was 221 bp. PCR products were then digested by endonuclease restriction enzyme, HhaI to digest 3’-end and Sau3AI to digest 5’-end. Genotype analysis results by RFLP shown in Table 3.

Until recently, there is no report of human T. gondii genotypes in Indonesia and whether certain genotype is related to certain clinical manifestation is still a debate. Several methods can be used to investigate T. gondii types, from isoenzyme electrophoresis, restriction fragment length polymorphism (RFLP), multiplex PCR, DNA microsatellite analysis, and DNA sequencing [5, 13, 14, 15]. Of all above, PCR-RFLP is the most common method used in more than 1,000 isolates in the world and had produced significant data on T. gondii genetic variation [16].

This work is part of a study on neuroaids where Toxoplasma laboratory screening has been
performed by PCR on B1 gene, resulted in 28/88 (31.82%) cerebrospinal fluid samples and 8/64 (12.5%) of aqueous humor were positive for *T. gondii*. The incidence of active cerebral toxoplasmosis in our study is higher than ocular toxoplasmosis, as well as to similar study on HIV infected patients in Malaysia, which was 11.3% [17].

Studies about prevalence of ocular toxoplasmosis in Asia showed various results. In India, the incidence varied from 1.7 – 12%, while in Japan, *T. gondii* is only responsible for 1.1% of uveitis cases; 8.7% of uveitis cases in Thailand were caused by *T. gondii* [18]. Mahalakshmi et al. (2010) also reported that B1 gene is generally more sensitive to detect *T. gondii* DNA compared to SAG2, though SAG2 is able to rapidly distinguish *T. gondii* genotype as it has polymorphic sites specific for each type [19].

Studies on genetic analysis of *T. gondii*, reported that amplification of SAG2 required only small amount of DNA sample, which make it possible to be applied directly on clinical samples [4]; thus, important information about various *T. gondii* strain from patients suspected from clinical toxoplasmosis can be analyzed [10]. The Surface Antigen Gene 2 (SAG2) codes *T. gondii* P22 protein, the main surface protein showing good antigenicity and immunogenicity characteristic. It is suitable for rapid genotyping because it has specific polymorphism for each type [19].

Our study showed that amplification of 5’-SAG2 gene of *T. gondii* was very good, resulted in 92.8% of 28 cerebrospinal fluid and 100% of 8 aqueous / vitreous humor were positive. The good result on 5’-SAG2 gene was not replicable for PCR of 3’-SAG2, which gave positive result in only 14.3% of 28 spinal fluids and 25% of 8 samples aqueous/vitreous humor previously positive on B1 screening. The failure to amplify locus SAG2 might be caused by the small amount of parasite in the samples [10]. The presence of polymorphism in primer attachment site of the studied isolates is also possible [20].

Amplicons of SAG2 gene, both 5’ and 3’ends were then subjected to RFLP to reveal the genotype based on digestion by Sau3AI and HhaI restriction enzymes. Sau3AI recognize restriction site on *T. gondii* type III, whereas HhaI enzyme can recognize restriction site on *T. gondii* type II. Samples which are not digested by both enzymes suggested the type I [10].

Genotyping by PCR-RFLP on cerebrospinal fluid samples could only be accomplished in 4 samples which were successfully amplified the 5’- and 3’ SAG2 genes, resulted as type I *T. gondii* (figure 3). The rest 22 spinal fluids which were PCR positive merely on 5’-SAG2, the RFLP could distinguish between type III and non-type III (I/II), resulted all to be non type III. No type III was found in the spinal fluid samples.

As genotyping of *T. gondii* from aqueous/vitreous humor could only be performed on 2 samples which completely amplified both SAG2 genes, resulted in type I. The rest 6 samples which only amplified 5’-SAG2 were found to be either type I or II strain. None of the aqueous/vitreous humor samples was of type III strain.

Various studies reported that type I was the most pathogenic strain in immunocompromised patients. A study in Brazil showed 46% patients were found to be infected by type I strain [6]. Similar results were also reported by Khan et al. (2005), among 11 samples obtained from HIV infected patients and diagnosed as *Toxoplasma* encephalitis, were infected by type I *T. gondii* [15].

Identification of *T. gondii* type in aqueous/vitreous humor in this study showed similar result as with the spinal fluids i.e. Type I or type II strains of *T. gondii*. This result is supported by prior study result in Brazil which found only type I strain in all 11 ocular samples positive for *T. gondii* [21]. There were also three other reports documenting isolation of *T. gondii* from immunocompetent adults with ocular toxoplasmosis. Two of the isolation, which were performed in mice were virulent towards mice, so it is likely type I, or at least not type II Nor type III, suggesting that type I strain is the most related to ocular toxoplasmosis in immunocompetent adults [7].

Nevertheless, the data above are contradictive

| Samples   | Type I (%) | Type I or II (%) | Type III (%) | Total (%) |
|-----------|------------|------------------|--------------|-----------|
| CSF       | 4 (15.38%) | 22 (84.62%)      | 0 (0%)       | 26 (100%) |
| Ocular fluid | 2 (25%)    | 6 (75%)          | 0 (0%)       | 8 (100%)  |

Table 3. Genotyping of *T. gondii* from spinal and ocular fluid

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My data does not show any specific pattern or trend that can be analyzed without context. The table above shows the genotyping results for different samples, but further analysis would be needed to determine specific patterns or trends.

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with the notion that type II is the most related to toxoplasmosis [22]. It is likely to be caused by limitation in sample analysis which used only one gene target to determine the parasite genotype, and it is strongly suggested that the strain which causes lesion in brain or eye is a mixed infection because the genetic difference amongst strain is indeed very little [21]. Genetically, the difference amongst each type is only 1 – 2%, but the virulence differs significantly [2]. SAG2 gene is useful in distinguishing type I, II, and III, but in this study, we did not find atypical strain as it is commonly found in Southern America [6]. Besides, characterization of genotype with gene SAG2 can be performed directly in clinical samples, thus it prevents bias in prevalence of certain type contributed by duplications of certain type in culture process [20].

Other aspect to be noted in direct genotyping is the failure in amplification of some samples using standard protocol as described by Howe et al (1997) [10]. It could be due to the presence of polymorphism in primer attachment sites among the Indonesian isolates, which are different geographically compared to other countries isolates [20].

**Conclusion**

Type I and likely type II are the most common strain found in *T. gondii* infection of brain and eye. This study is the first research on genotyping of *T. gondii* strain with clinical manifestation and transmission pattern in Indonesia.

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