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Research Articles

Nested PCR method for detection *Toxoplasma gondii* B1 gene in Cerebrospinal Fluid of HIV patients

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Abstract

Background: Toxoplasmosis is a disease caused by infection of *Toxoplasma gondii* which may cause a life-threatening condition in immunocompromised patients, for example, *Toxoplasma* encephalitis (TE). It is challenging to diagnose *Toxoplasma* as a cause of central nervous system (CNS) infection in HIV patient, so we need an alternative method, which is a PCR detection of *Toxoplasma gondii* B1 gene.
Objective: This research aimed to find association between PCR methods for *Toxoplasma gondii* B1 gene and anti-*Toxoplasma* IgG from cerebral spinal fluid patient HIV AIDS.
Methods: A cross-sectional study was conducted to Cerebrospinal fluid (CSF) samples of HIV patients with neurological symptoms to determine *Toxoplasma gondii* infection using nested PCR methods for the B1 gene and detection of anti-*Toxoplasma* IgG.
Results: 88 CSF samples from HIV patients tested using nested PCR showed 23 samples (26,1%) were positive. Serologic test for IgG *Toxoplasma* showed 34 samples were positive (28,6%). There was a significant correlation (p=0.000(<0.05) between PCR result and a serologic test for IgG *Toxoplasma*.
Conclusion: Nested PCR methods to detect B1 gene increased the accuracy of diagnosis for *toxoplasma* encephalitis.

Keywords: *cerebrospinal fluid; Toxoplasma gondii; PCR; IgG anti Toxoplasma; HIV*
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INTRODUCTION

Toxoplasma gondii infection comes from the protozoan parasite, which is predicted to infect about one-third of the world's population.[1] The prevalence of toxoplasmosis in Jakarta is estimated to reach 70%.[2] In normal condition, *T. gondii* infection is asymptomatic, but in some immunocompromised individuals, a bradyzoite-containing cyst can rupture and reactivate the latent infection-causing *toxoplasma* encephalitis (TE).[3] *Toxoplasma* encephalitis (TE) attacks the central nervous system (CNS), especially in patients with severe HIV infection, even though the incidence of this disease decreases because of antiretroviral drugs. It is predicted that about 10-50% of AIDS patients with toxoplasmosis will continue to be TE.[4] In Indonesia, TE is still the main CNS disorder in AIDS patients followed after Tuberculous meningitis.[5]

A definitive diagnosis of TE can be provided by brain biopsy and finding *T. gondii* tachyzoites. The examination is an invasive procedure and associated with a significant increase in morbidity and mortality. A tentative diagnosis of this disease only based on CNS signs and symptoms, the positive result of anti-*T. gondii* antibody and typical single lesions or multiple ring-enhancing lesions in the brain detected by Computed Tomography (CT) and Magnetic Resonance Imaging (MRI).[1,6] Disadvantages of serologic test for *Toxoplasma* were the serologic result of anti-*Toxoplasma* antibody should be interpreted carefully and it can be failed to detect anti-*Toxoplasma* antibody in the early phase of infection.[7,8] Serologic test also unable to detect *Toxoplasma* infections in immunocompromised patients,

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because there is a disturbance in immunoglobulin production, where anti-*Toxoplasma* IgG only appear in 30% HIV patients with active toxoplasmosis, and only 2% shows positive for IgM.[9,10]

The molecular diagnosis using PCR for various clinical specimens become a powerful tool for detection of *T. gondii* DNA.[9,11] The problem is the sensitivity of this technique is not yet established.[7,12] The utilization of PCR for detecting *T. gondii* in CSF has been done. PCR can detect *T. gondii* DNA using primer for the B1 gene, which is frequently used and has remarkable sequence conservation (99.5%) of the B1 gene. It also has been confirmed by analysis of DNA sequences from many clonal strains.[9] Some studies found that PCR sensitivity to detect B1 gene is 83.3%, and its specificity reached 95.7%[12], and another experiment shows 100% sensitivity and 94.9% specificity[13].

The aim of the present study is to find association between of PCR methods for *Toxoplasma gondii* B1 gene and anti-Toxoplasma IgG from cerebral spinal fluid patient HIV AIDS.

MATERIALS AND METHODS

This study was held in the Laboratory of Parasitology Faculty of Medicine Universitas Indonesia, November 2015 - January 2016. A cross-sectional study was performed to determine *Toxoplasma gondii* infection from cerebrospinal fluid (CSF) of HIV patients with neurological symptoms. The inclusion criteria were all cerebrospinal fluid samples of HIV patients with neurological symptoms available in the Laboratory of Parasitology Faculty of Medicine Universitas Indonesia. The sampling technique used in this study was consecutive sampling, which means every CSF was included as a sample until minimal samples were achieved.

DNA isolation from CSF and Amplification of targeting DNA

DNA from CSF samples were extracted using DNA mini kit the QIAGEN method with 80μL AE buffer. *T. gondii* was detected using B1 gene amplification. The amplification was performed individually in 20μL of reaction mixture containing 1x PCR buffer, 1.5 mM MgCl2, 200 μM each were dNTP (dATP, dGTP, dTTP, dCTP), 250 μM for each primer, and 1.5 U Taq polymerase. The other conditions were optimized carefully before the study was started. Primers used were the same primers as the previous study[14] in gene amplification, as seen below.

PCR Round	Forward PCR primers (5' -3')	Reverse PCR primers (5'-3-)
First	GGAACTGCATCCGTTCATGAG	GGCGACCAATCTGCGAATACACC
Second	TGCATAGGTTGCAGTCACTG	TCTTTAAAGCGTTCGTGGTC

As many as 3 μl extracting DNA from CSF samples were added to the mixture to make the template for the first PCR round, followed by 2 μl DNA results from the first PCR round was used as templates for the second PCR round. The reaction condition was modified from previous study to optimize the PCR condition.[12] PCR steps to amplify target gene were: initialization step, which consisted of heating temperature at 95ºC for 7 minutes. Denaturation step continued as heating the reaction to 95ºC for 1 minute, annealing step at 48ºC for 1 minute, and followed by extension by extension step in 72ºC for 2 minutes, and repeated for 35 times/cycles. The reaction was ended at 72ºC extension for 7 minutes.

Amplification result was separated using agarose gel electrophoresis in 0.5x TBE (0.05M Tris Base, 0.05M borate acid, and 0.001 M pH 8.5 EDTA) buffer and stained with SYBR green. Electrophoresis was run at 100 volts for 50 minutes, and then the result was viewed under UV light.

Each PCR reactions included one negative control (pure water) and one positive control (from DNA extraction of *T. gondii* tachyzoites).

IgG anti-*Toxoplasma* Examination from CSF

IgG anti-Toxoplasma examination uses the Anti-*Toxoplasma gondii* ELISA (IgG) EUROIMMUN ™ kit (Cat number EI 2410-9601G, Lűbeck, Germany) and follows the procedure listed in the kit.

Statistical analysis

The result of PCR was categorized as positive or negative, and the result of serologic titer for IgG-*Toxoplasma* was divided into three groups based on Derouin et al.[15] Those groups were negative (<11IU), low titers (≥11-150IU) and high titers (>150IU). These two different methods to be statistically analyzes using Chi-Square test for trend.

Ethical aspect

Ethical approval for the study was obtained from The Ethics Committee of Faculty of Medicine, University of Indonesia, which approved all protocol in this study.

RESULTS

This study using CSF samples from HIV patients with neurological disorders for example meningitis. There were 88 CSF available from Parasitology Department Faculty of Medicine Universitas Indonesia for examination. As many as 49 samples came with clinical diagnosis i.e. Tuberculosis meningoencephalitis (TB ME, 46%), *Toxoplasma* encephalitis or TB ME (28%), Cryptococcus meningitis or TB ME (12%) and no clinical diagnosis for the rest. The samples originated from 90% male and 5% female with age ranged 20 to 50 years old.

PCR examination results.

B1 gene detection using nested PCR method had shown that 23 out of 88 samples were positive. Positive results were characterized by obtaining a specific band of DNA *Toxoplasma gondii* at 131 bp whereas negative result showed no band at all (**Figure 1**).

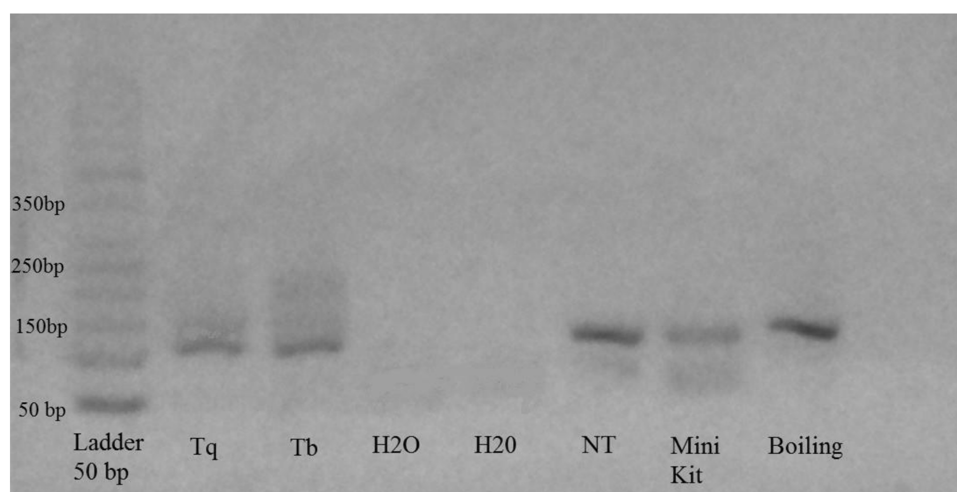

Figure 1. Optimization of DNA extraction results. From left to right: ladder 50bp, tachyzoites extraction using QIAGEN minikit method (Tq), boiling method for tachyzoites (Tb), negative control 1 (H20), negative control 2 (H20), direct method for CSF (NT), minikit method for CSF (Mini kit), boiling method for CSF (boiling)

Table 1. Comparison between PCR-based method and serologic test for IgG anti-*Toxoplasma* from CSF

		Serologic test for IgG anti *Toxoplasma*						Total	%
		Negative	%	Low Titer IgG	%	High Titer IgG	%		
PCR results	Positive	4	7,4%	12	48 %	7	77.8%	23	26.1
	Negative	50	17%	13	56.8%	2	22.22%	65	73.9
	Total	54	100%	25	100 %	9	100%	88	100

Negative titer below: 11 IU, Low titer IgG anti *Toxoplasma*: ≥11-150 IU and high titer IgG anti *Toxoplasma* >150 IU

PCR examination and serologic test using ELISA

Table 1 shows there were 23 CSF samples (26.1%) positive by nested PCR of the B1 gene while the anti-*Toxoplasma* IgG assay showed 34 samples positive (38,6%). Statistical analysis using Chi-Squared Test revealed a significant association ($p=0.000$, $p<0.05$) between the two methods and a significant positive correlation between PCR and IgG results (Spearman correlation =0.537 and $p<0.001$)

A higher proportion of positive PCR among those with higher anti-*Toxoplasma* IgG; 4 out of 54 (7.4%) samples negative for anti-*Toxoplasma* IgG, 12 out of 25 (48%) samples with low anti-*Toxoplasma* IgG and 7 out of 9 (77.7%) samples with high anti- *Toxoplasma* IgG. It was clear that positive PCR results had a trend to associate to higher IgG concentration, as well as the negative PCR results to very low and negative IgG titer.

DISCUSSION

In this study, the nested PCR for *Toxoplasma gondii* B1 gene could be alternative to diagnose *Toxoplasma* encephalitis in HIV patients. This study showed that positive samples showed higher titer of anti-*Toxoplasma* IgG.

Nested PCR was chosen as a method in this research, due to nested PCR had a respectable sensitivity.[16–18] Choosing the B1 gene as a target was due to its amplification has some advantages, such as higher sensitivity than another targeting gene.[19] B1 gene did not amplify DNA from any other bacterial and fungal, and its sensitivity was not changed because of different DNA count in human and increase in protein. This gene also had good gene conservation.[20] Moreover, the B1 gene was the most often gene used in toxoplasmosis molecular study.[12–14]

From 88 samples in this study, 26.1% showed positive PCR results. This finding supported the previous experiment that the sensitivity to diagnose TE (*Toxoplasma* encephalitis) from CSF was about 17—100%.[12] It should be noted that PCR sensitivity was relied on some factors like the chemical and physical condition of reaction, targeting DNA concentration, choosing primer, and DNA extraction method. In this study, there was a limitation in sample volume, which was a little that could affect the DNA concentration then affect the sensitivity of the test.[21] Nevertheless, this data showed that the PCR procedure in this study could be used to diagnose *Toxoplasma* encephalitis.

IgG was selected as the primary test to detect *Toxoplasma* infection because most of the HIV patients with CNS disorder originated from reactivation of past Toxoplasma infection. The previous study also confirmed the predominance of IgG antibodies in immune response to *T.gondii*.[22] To assess the active infection stage, molecular detection by PCR was chosen instead of anti-*Toxoplasma* IgM which may not be produced due to the immunocompromised state.

IgG examination results showed a higher proportion of patients positive for anti-*Toxoplasma* IgG in comparison to the PCR result (38.6 % vs 26.1%) suggesting that most patients suffered from *Toxoplasma* reactivation. This high positive percentage did not mean

that the patients were actively infected, as a small proportion of patients with negative anti *Toxoplasma* IgG, showed positive PCR result. *Toxoplasma* serology test could fail to detect anti-*Toxoplasma* antibody especially in the early phase of primary *Toxoplasma* infection. *Toxoplasma* serology test could fail to detect anti-*Toxoplasma* antibody especially in the early phase of primary infection. This situation might be due to the antibody that was not produced until several weeks of parasitemia exceeded.[3]

This result is also consistent with the previous study[4]. That study used the same targeting gene as this study. The difference was the initial CSF volume and PCR method used. Ganiem et al. used 7 ml CSF and real-time PCR method. Another difference was the specimen used. In this study, we used CSF as samples, but the previous one used serum from HIV patients as samples.

This research came with the result that positive PCR had a trend to show higher IgG concentration. The statistical analysis was showing the significant positive correlation between PCR test and IgG status. Other investigation also showed similar results[4]. Nevertheless, it should be noted that in this research, several samples that show higher titer with negative PCR results.

The use of PCR will tell us the active stage of *Toxoplasma* infection while measuring one time anti Toxoplasma IgG will not be able to tell active infection, however we tried to find any correlation between those two tests and got R=0.537 suggesting at some point IgG anti-*Toxoplasma* may associate to active infection however there is a need for a second test to confirm it, and PCR is currently the best. According to Vidal JE et al, all patients with cerebral toxoplasmosis presented positive result of PCR B1 (sensitivity 100% and specificity 94.4%) from CSF sample while poor performance was obtained when using blood samples (100% specificity and 25% sensitivity). [13,23]

The limitation in this study, beside the lack of sample volume, several samples were the old collections saved for a long time. Samples in this study were part of previous study on detection of Cryptococcus in neuroAIDS and have undergone the freeze-thawing process for two times. As very small left over of the cells, this may also cause negative PCR result. This sample condition resulted in DNA degradation and caused several IgG results with high titer could not be detected by PCR. It is consistent with the previous study that reached 33.33% sensitivity from PCR. The sensitivity was increasing up to 50% if lumbal puncture was done one week before therapy and directly examined.[21,24]

From these results of *Toxoplasma* PCR examination from CSF, it was clearly stated that the high sensitivity could rule out other differential diagnosis. This test could be done to complete other supporting tests such as radiology, serology examination to diagnose active infection of *Toxoplasma* encephalitis.

A more complex experimental design is needed to include several HIV and AIDS centers and more samples to find the significant correlation between variables. Future study between PCR, serology of anti-*Toxoplasma* IgG and clinical information is needed and should involve several HIV AIDS centers with larger number of samples.

CONCLUSION

Nested PCR of B1 gene *Toxoplasma gondii* from CSF could be used to diagnose *Toxoplasma* Encephalitis in HIV patients. The positive PCR results tend to have higher anti-*Toxoplasma* IgG titer. This study showed that PCR methods to detect B1 gene was helpful for increasing the accuracy of diagnosis for *Toxoplasma* Encephalitis.

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