Detection of *Toxoplasma gondii* and Epstein-Barr virus in HIV patients with clinical symptoms of suspected central nervous system infection using duplex real-time polymerase chain reaction

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Abstract. Focal brain lesion is a neurological complication in HIV, which is marked as a space occupying lesion (SOL) and needs rapid and effective treatment. This lesion is mainly caused by encephalitis toxoplasma and primary central nervous system lymphoma related to the Epstein-Barr virus (EBV) infection, which is difficult to distinguish using CT scan or magnetic resonance imaging (MRI). The gold standard of diagnosing focal brain lesion has been brain biopsy, but this examination is an invasive procedure that causes complications. The objective of this study is to obtain the rapid laboratory diagnosis of *Toxoplasma gondii* (*T. gondii*) and EBV infection. In this experimental study, blood and cerebrospinal fluid were obtained from HIV patients who were admitted to the Neurology Department of Cipto Mangunkusumo Hospital. The samples were examined using duplex real-time polymerase chain reaction (PCR) to detect *T. gondii* and EBV. The first step was the optimization of duplex real-time PCR, including the annealing temperature, primer and probe concentration, elution volume, and template volume. Minimal DNA detection was used to measure minimal *T. gondii* and EBV. Cross reactions were determined for technical specificity using the bacteria and viruses *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* H37Rv, *Candida spp*, cytomegalovirus, herpes zoster virus, and varicella zoster virus. Duplex real-time PCR was applied optimally to patients. In the optimization of duplex real-time PCR, the annealing temperature of *T. gondii* and EBV were 58 °C, the concentration of primer forward and reverse for *T. gondii* and EBV were 0.2 µM, the concentration of probe for *T. gondii* and EBV were 0.4 µM and 0.2 µM, respectively. Minimal DNA detection of *T. gondii* and EBV were 5.68 copy/ml and 1.31 copy/ml, respectively. There was no cross reaction between another bacteria and virus that were used as the primer and probe for *T. gondii* and EBV. The blood duplex real-time PCR was positive for *T. gondii* (16%), EBV (40%), and both (16%). The cerebrospinal fluid samples were positive for *T. gondii* (20%), EBV (28%), and both (4%).

1. Introduction
In HIV infection, neurological complications are frequent. Before antiretroviral therapy, such complications occurred in 7–20% of HIV patients [1]. Focal brain lesion in HIV patients is an important issue that requires rapid and appropriate treatment to save the patient’s life. The diagnosis of brain...
lesions in HIV patients is still a debatable issue. In HIV patients, 30–40% showed an impaired central nervous system, and 10% of patients with central nervous system disorder showed one or more brain lesions on examination \([2,3]\). Previous research found that in developing countries, focal brain lesions in HIV patients may be caused by opportunistic infections, malignant disease, and cerebrovascular disease, which are life-threatening \([2,3]\). Focal brain lesions in HIV patients are characterized by SOLs in radiological examination. The differential diagnosis of focal brain lesions may be caused by primary brain lymphoma, toxoplasma encephalitis, progressive multifocal leukoencephalopathy (PML), HIV encephalopathy, encephalitis caused by cytomegalovirus, encephalitis caused by the herpes simplex virus, tuberculoma, cryptococcoma, vasculitis and abscess. Approximately more than 50% of the causes of brain lesions in HIV patients are toxoplasma encephalitis and primary brain lymphoma \([1,4]\).

This study is a part of a larger research conducted at Cipto Mangunkusumo National Hospital (RSCM), which focuses on identifying the causes of brain infection in HIV patients. The identification of the causes of these brain infections includes the detection of \textit{Toxoplasma gondii} (\textit{T. gondii}), Epstein-Barr virus (EBV), Cryptococcus neoformans, Mycobacterium tuberculosis, and cytomegalovirus. As part of this research, this study contributes to developing a real-time PCR duplex test to detect \textit{T. gondii} and EBV. In some countries, the differential diagnosis of toxoplasma encephalitis is primary brain lymphoma. Malignancy in the brain is associated with an infection caused by EBV, and it occurs in patients with CD4 less than 100 cells/mm\(^3\) \([3]\). Primary brain lymphoma can occur in approximately 3% of patients diagnosed with brain tumors and in 2–3% of all cases of non-Hodgkin lymphoma \([5]\). The Epstein-Barr virus plays a role in the process of primary brain lymphoma. If this viral DNA is found in the cerebrospinal fluid of HIV patients, it is a sign of primary brain lymphoma infection \([6]\).

In toxoplasma encephalitis, radiological features are characterized by multiple nodules or ring lesions with edema and the effects of duration. Of the lesions of toxoplasma encephalitis identified in CT scans tests, 27% were single lesions. Only 14% of MRI tests showed a single lesion. Most lesions are present in the basal ganglia, the frontal lobe, and the parietal lobe. On CT scan, the lesions in primary brain lymphoma showed either hyperintense or isointense lesions that were surrounded by edema and amass with increasing homogeneous contrast. Most lesions are multifocal and located in the periventricular \([4,7]\).

Toxoplasma encephalitis and primary brain lymphoma are similar, and they are difficult to distinguish both clinically and by radiological examination. The definitive diagnosis is by brain biopsy, but it is rarely done because of its neurological complications. Anti-toxoplasma empiric therapy is usually used to distinguish toxoplasma encephalitis and primary brain lymphoma. This therapy is performed for 14 days, and it often delays the diagnosis and treatment of patients with this complication. Thallium brain SPECT examination can be used to distinguish toxoplasmosis and primary brain lymphoma causing brain mass lesion in less than 72 hours \([8,9]\). Polymerase chain reaction (PCR) is a molecular examination that can be used to detect the presence of \textit{T. gondii} and EBV in brain tissue, cerebrospinal fluid (CSS), vitreous and aqueous fluid of the eyes, bronchoalveolar saline solution, amniotic fluid and blood in acquired immune deficiency syndrome (AIDS) patients \([10]\). Several studies found that the PCR examination of cerebrospinal fluid clinical specimens could replace a definitive diagnosis by brain biopsy with better sensitivity and specificity \([10-12]\). Positive PCR results in cerebrospinal fluid are a strong indicator for the diagnosis of toxoplasma encephalitis \([13]\). In addition to the high level of specificity, this method has the advantage of not being affected by the immune response capability, so detection can be performed at each phase of the disease \([13]\). Real-time PCR is the preferred method because it can provide a precise and rapid diagnosis of the reactivation of toxoplasmosis in immunocompromized patients \([14-16]\). This study used the PCR method to detect \textit{T. gondii} as the cause of toxoplasma encephalitis and EBV as the cause of primary brain lymphoma in HIV patients with neurologic complications. In this study, blood and cerebrospinal fluid specimens were examined. The results of this study showed that blood and cerebrospinal fluid can be used to detect \textit{T. gondii} and EBV, thereby reducing the invasive action that is often contraindicated for patients with increased intracranial pressure.
2. Materials and Methods

This experimental study was conducted at the Laboratory of Virology and Molecular Biology, Department of Microbiology, Faculty of Medicine, Universitas Indonesia. Samples were taken after the study proposal was approved by the Medical Research Ethics Committee of the Medical Faculty of Universitas Indonesia. The subjects were HIV patients with suspected brain infection, who were treated in the Neurology Department of Cipto Mangunkusumo National Hospital (RSCM), Jakarta. The inclusion criteria were HIV patients older than 18 years with clinical symptoms of brain infection (at least two of the four common neurological symptoms, which are fever, headache, loss of consciousness) with or without liver failure, and not having received any treatment. The exclusion criteria were pregnancy with increased intracranial pressure and unwillingness to take part in the study.

All samples were taken from blood and cerebrospinal fluid, which then were stored at -80 °C until they were used. Real-time PCR duplex optimization at annealing temperatures, primary concentrations and probes of *T. gondii* and EBV, cross-reaction tests against microorganisms other than *T. gondii* and EBV were conducted. A DNA detection threshold limits test was also performed. Then the blood samples and cerebrospinal fluid of HIV patients with clinical symptoms of suspected brain infection were tested using real-time PCR duplex. The mechanism of the work was the DNA extraction of blood samples and cerebrospinal fluid using the QIAamp DNA minikit (Qiagen). A Real-time PCR duplex test was developed to detect *T. gondii* and EBV in the samples of blood and cerebrospinal fluid. To obtain sensitive detection tests, several parameters were optimized at this stage, including the annealing temperatures, primary concentrations, EBV probes, *T. gondii*, inhibitor test, specificity test (cross reaction), and sensitivity test.

3. Results and Discussion

3.1 Results

3.1.1. Optimization of Annealing Temperature

This optimization used an annealing temperature gradient of 54–64 °C. The reaction composition consisted of a 10-μl kappa fast probe, 0.2 μM primary forward, *T. gondii* and EBV reverse, 0.4 μM *T. gondii* probe, and 0.2 μM EBV probe. The reaction composition consisted of a 10-μl kappa fast probe, 0.3-μM *T. gondii* probe, 0.2 μM EBV, 3-μl positive control of *T. gondii* and EBV, and distilled water to a maximum volume of 20 μl for one reaction, and it was performed twice. Positive results were determined by the number of fluorescent signals read by the PCR real-time filter engine. The temperature gradient result showed thermal cycles at 95 °C for 3 minutes and 95 °C for 15 seconds. The optimum annealing temperature of *T. gondii* was 60 °C for 1 minute with a 45x cycle. Figure 1

![Figure 1](image)

**Figure 1.** *T. gondii* annealing temperature optimization results using real-time PCR duplex*Orange line: 60 °C; Light Blue Line: 58 °C; Black Line: 55 °C; Yellow Line: 62 °C; Old Blue Line: 64 °C; Line of Green Color: 63 °C; Blue Line: 56 °C; Red Line: 54 °C
shows the optimal *T. gondii* annealing temperature of 60 °C. Figure 2 shows the optimal annealing temperature of the Epstein-Barr virus at 58 °C. In real-time PCR duplex, the annealing temperature obtained for both was 58 °C, which indicated the optimal temperature.

**Figure 2.** Epstein-Barr virus annealing temperature optimization results using real-time PCR duplex.*1. Black: 58 °C; 2. Orange: 60 °C; 3. Green: 62 °C; 4. Light Blue: 54 °C; 5. Gray: 56 °C; 6. Red: 63 °C; 7. Dark Blue: 64 °C

3.1.2. Optimization of Primary Concentration

The concentrations of forward and reverse primers were tested in this study. The results of the primary optimization obtained in this research were 0.1 μM, 0.2 μM, 0.3 μM, and 0.4 μM with a total volume of 20 μl. The positive controls used by EBV were derived from positive DNA extraction with conventional PCR testing and *T. gondii* using synthetic DNA. The reaction composition consisted of a 10-μl kappa fast probe, a 0.3 μM *T. gondii* probe, 0.2 μM EBV, 3 μl positive control of *T. gondii* and EBV, and distilled water to a maximum of 20 μl for one reaction, and it was performed twice (duplo). Figures 3 and 4 show the optimal signal amplification results of 0.2 μM concentrations for *T. gondii* and the Epstein-Barr virus.

**Figure 3.** Optimization of *T. gondii* primary concentrations*1. Black lines: 0.2 μM, 2. Red lines: 0.3 μM, 3. Green lines: 0.4 μM, 4. Blue lines: 0.1 μM
3.1.3. Optimization of Probe Concentration

The probe concentrations tested in this study were 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM. The probe used by *T. gondii* is a labeled DNA fragment. Its nucleotide sequence was identified in Kompalic et al’s study as 5’ (6-FAM)-CCACCTCGCCTCTTGG- (NFQ-MGB) 3’. The probe used by EBV was identified in Sugita et al as 5’FAM-TGTACACGCACGAGAAATGCGCC-TAMRA 3’. The reaction composition consisted of a 10-μl kappa fast probe, 0.2 μM primary and reverse primers, 3 μl positive and distilled water controls to a maximum volume of 20 μl for one reaction, and it was performed twice (duplo). The optimal probe concentration conditions obtained were 0.4 μM for *T. gondii* and 0.2 μM for EBV.

Figure 4. Optimization results of the Epstein-Barr virus primary concentration.*1. Black lines: 0.2 μM, 2. Red lines: 0.4 μM, 3.Green lines: 0.3 μM, 4. Yellow lines: 0.1 μM

Figure 5. *T. gondii* test results using various probe concentrations*1. Black lines: 0.4 μM; 2. Yellow lines: 0.3 μM; 3. Red lines: 0.2 μM; 4.Gold lines: 0.1 μM
Figure 6. Epstein-Barr virus test results using various probe concentrations*1. Black line: 0.2 μM; 2. Red line: 0.4 μM; 3. Blue line: 0.5 μM; 4. Orange line: 0.3 μM; 5. Green line: 0.1 μM

3.1.4. T. gondii and EBV Specificity Test (Cross-Reaction Test)
Real-time PCR tests were performed on T. gondii and EBV to determine the primary specificity and probe that were obtained previously. This method cannot cross-react with the genomes of other microorganisms. This specificity test uses several microorganisms that can be detected in blood and cerebrospinal fluid, such as Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Mycobacterium tuberculosis H37Rv, Candida spp, cytomegalovirus, herpes zoster virus, and varicella zoster virus. Bacterial DNA was isolated from culture using the QIAamp DNA kit Blood Mini Kit (Qiagen). Viral DNA was derived from the extraction of a positive specimen. The reaction composition consisted of a 10-μl kappa fast probe, T. gondii and reverse 0.2 μM reverse primers, a 0.4-μM T. gondii probe, an EBV 0.2-μM probe, a 2-μl DNA template, and the DNA mold of a positive specimen; 5 μl of distilled water was added to a maximum volume of 20 μl.

Figure 7. PCR real-time specificity test results showed that Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Mycobacterium tuberculosis H37Rv, Candida spp, cytomegalovirus, herpes zoster virus, varicella zoster virus were not cross-reacted with primers used for T. gondii and EBV detection *1. Pink curve: Pseudomonas aeruginosa; 2. Young green curve: Staphylococcus aureus; 3. Light blue curve: Klebsiella pneumonia; 4. Dark green curve: Mycobacterium tuberculosis H37Rv; 5. Red curve: Candida spp; 6. Orange curve: cytomegalic- virus; 7. Old blue curve: herpes zoster virus; 8. Brown curve: varicella zoster virus
3.1.5. Sensitivity Test (DNA Detection Threshold)
This test was performed to determine the lowest detectable DNA concentrations. The reaction composition consisted of a 10-μl kappa fast probe, *T. gondii* and reverse 0.2 μM reverse primers, 0.4-μM *T. gondii* probe, EBV 0.2 μM probe, and a 1-μl DNA mold of serial dilution. Distilled water was added to a maximum volume of 20 μl. The lowest DNA concentration that gave the optimum amplification result was 3.8x10^-12 ng/μl (3.8x10^-7 fg/μl) and 5.68 copy number/ml. The lowest DNA concentration that gave the optimal amplification result was 14x10^-13 ng/μl (14x10^-7 fg/μl) and 1.31 copy number/ml.

**Figure 8.** Results of real-time PCR at some concentrations of *T. gondii* DNA dilution. *1. Red line: 3.8x10^-10 ng/μl; 2. Black line: 3.8x10^-11 ng/μl; 3. Yellow line: 3.8x10^-12 ng/μl; 4. Blue line: Negative controls

**Figure 9.** Results of real-time PCR at some concentrations of EBV DNA dilution *1. Orange line: 14x10^-11 ng/μl, 2. black line: 14x10^-12 ng/μl, 3. green line: 14x10^-13 ng/μl, 4. yellow line: negative control

In this study, the characteristic data of HIV patients with brain infection showed that the highest number of brains infections in HIV were male patients at as high as 76%, and in the age range of 25–35 years as high as 48%. The highest CD4 cell count in HIV patients with a brain infection suspect was CD4 < 50 cells/μl of 68%. Most major complaints (80%) were for headache. The CT scans showed brain focal lesions in 56% of these patients.
Table 1. Characteristics of HIV patients with brain infection (n= 25). Patient data are taken from medical record data

| Characteristics          | n   | Percentage (%) |
|--------------------------|-----|----------------|
| Sex                      |     |                |
| Male                     | 19  | 76             |
| Female                   | 6   | 24             |
| Age                      |     |                |
| <25 years old            | 2   | 8              |
| 25-35 years old          | 12  | 48             |
| 35-45 years old          | 8   | 32             |
| >45 years old            | 3   | 12             |
| CD4 Counts               |     |                |
| <50 cells/μl             | 17  | 68             |
| 50-100 cells/μl          | 6   | 24             |
| >100 cells/μl            | 2   | 8              |
| Major Complaint          |     |                |
| Headache                 | 20  | 80             |
| Loss of consciousness    | 13  | 52             |
| Limb weakening            | 9   | 36             |
| Fever                    | 9   | 36             |
| Seizures                 | 4   | 16             |
| Oral candidiasis         | 5   | 20             |
| Cryptococcus             | 2   | 8              |
| CT Scan                  |     |                |
| Focal lesion             | 14  | 56             |
| No focal lesion          | 8   | 32             |

The results of the analyses of blood and CSS samples in this real-time PCR duplex study are shown in Figure 10. The results showed that the biggest cause of brain infection in HIV patients was caused by the Epstein-Barr virus. The virus was found 40% of the blood samples and in 28% of the *T. gondii* cerebrospinal fluid samples; the virus was detected in 16% of the blood samples and in 20% of the cerebrospinal fluid samples. Positive results of *T. gondii* and EBV were found in 16% of the blood samples and in 8% of the cerebrospinal fluid samples.

![Real-Time PCR Result of CSF and Blood Sample Tests](image)

Figure 10. Real-time PCR results of blood and cerebrospinal fluid sample tests
3.2. Discussion

3.2.1. Optimization of Real-time PCR Duplex
The optimization of real-time PCR duplex conditions showed that the annealing temperature of *T. gondii* was 60 °C. The temperature obtained is similar to that in Kompalic *et al.* [11], where the annealing temperature of *T. gondii* was 60 °C. In this study, the optimization the EBV annealing temperature was 58 °C. Therefore, the optimum temperature for both was 58 °C. The annealing temperature was determined based on the lowest Ct value (cycle threshold) and the highest fluorescent signal compared to other annealing temperatures. Primary and target gene used to detect *T. gondii* in this study was the B1 gene. Several studies have been conducted to compare the B1 gene and RE gene using real-time PCR. In Taqman, Mesquita *et al.* [17] study, the sensitivity value of the B1 gene was 86%, and the sensitivity value of the RE gene was 98%; the specificity value of the B1 gene was 97% and the specificity value of the RE gene was 88.8%. Cardona *et al.* [18] showed that 94.8% of the B1 gene was able to detect *T. gondii* in the blood, while the RE gene detected 87.2%. In a real-time PCR study with Taqman, Compalic *et al.* [11], compared the results of serological tests; the positive B1 gene and positive Ig M had a specificity value of 95.3% and a sensitivity value of 48.6%. The B1 gene is widely used as a target gene because it is specific indetecting *T. gondii* in blood and cerebrospinal fluid samples [11,17,18].

3.2.2. Specificity Test of Real-time PCR Duplex Technique
The results of the primary cross-reaction test and the PCR real-time duplex technique probe against other microorganisms showed that Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Mycobacterium tuberculosis H37Rv, Candida spp., cytomegalovirus, herpes zoster virus, and varicella zoster virus did not cross-react with the primary used to detect *T. gondii* and EBV. Joseph *et al.* also showed that Sarkocystis spp., Neospora spp., Plasmodium spp., Aspergillus spp., Candida spp., and Cryptococcus spp. did not cross-react to the primers used to detect *T. gondii*. Felner *et al.* [20] showed that the herpes simplex virus, cytomegalovirus, varicellazoster virus, and human herpesvirus6 did not cross-react to the primers used by EBV [19,20]. The results of this study showed no cross reactions to existing bacteria and viruses.

3.2.3. Sensitivity Test of Real-time PCR Duplex Technique
In this study, the real-time PCR duplex obtained a minimal DNA detection result in *T. gondii*, which was 3.8 x 10-12 ng/μl equivalent to the copy number of 5.68 copy/ml, and in the Epstein-Barr virus, which was 14x 10-12 ng/μl equivalent to the copy number of 1.31 copy/ml. In other studies, such as those reported by Hui Lin M *et al.*, the minimal detection of *T. gondii* was 0.05–5,000 copy number/ml. Gartzonika et al obtained a minimum DNA detection threshold value of EBV at 5.02 × 103 copies/ml [21,22]. Based on the results, this test was categorized as sensitive compared to other research results.

3.2.4. Characteristics of HIV Patient with Suspected Clinical Symptom Brain Infection
The results of the CT scans showed focal lesions on *T. gondii* (20%), Epstein-Barr virus (28%), and infection caused by both (4%). These results were in accordance with findings in Modi *et al.*, where the images of sting in the CT scans of HIV patients were associated with focal brain lesions (41.7%). These results illustrates that the stinging of CT scan results in patients with brain infections caused by the Epstein-Barr virus and *T. gondii* are difficult to distinguish [23].

3.2.5. Real-time PCR Duplex Results
Regarding the blood samples, 40% were positive for EBV and 16% were positive for *T. gondii*. In the cerebrospinal fluid samples, 20% were positive for *T. gondii*, 28% were positive for the Epstein-Barr virus, and 8% were positive for both the Epstein-Barr virus and *T. gondii*. This finding is consistent with the results of a study conducted by Mesquita *et al.*, which used real-time PCR against blood
samples and cerebrospinal fluid taken from HIV patients with suspected toxoplasma encephalitis using the B1 gene as the target. In that study, 34% of *T. gondii* was detected in the blood samples, and *T. gondii* was detected in 23.91% of the cerebrospinal fluid samples [16]. Serologic examinations for anti-toxoplasma are commonly used to diagnose toxoplasma encephalitis, but this examination has limitations. In HIV patients with decreased immune systems, the production of specific antibodies is disrupted, so false-negative results may be obtained [24]. Approximately 50% of *T. gondii* seroprevalence occurs in healthy HIV patients, whereas IgG is increased by 30% in HIV patients during toxoplasmosis infection [25]. Therefore, serology tests are not recommended to be used as a support for diagnosis regardless of clinical symptoms and radiological examination results. The limitation of this study is that a conclusion regarding the cause of brain infection in HIV patients that is consistent with the results of the examination cannot be drawn because no tests were performed to confirm the results of a real-time PCR duplex examination.

4. Conclusion

The optimal conditions of *T. gondii* and EBV detection tests using real-time PCR duplex were an annealing temperature of 58 °C, a primary concentration of *T. gondii* and Epstein-Barr virus of 0.2 μM, a *T. gondii* probe concentration of 0.4 μM, and an Epstein-Barr virus of 0.2 μM. The DNA mold for *T. gondii* and EBV was 5 μl. The specificity test of the PCR real-time technique showed that *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis H37Rv*, *Candida spp*, cytomegalovirus, herpes zoster virus, and varicella zoster virus did not cross-react with the primers used for the detection of *T. gondii* and EBV. The real-time PCR duplex detection threshold was created to detect the minimal amount of *T. gondii* DNA. The threshold was 3.8 x 10-12 ng/μl (3.8 x 10⁻¹² fg/μl), which was equivalent to 5.68 copy number/ml. The threshold of the Epstein-Barr virus was 14 x 10⁻¹² ng/μl (14x 10⁻⁴ fg/μl), which was equivalent to 1.31 copy number/ml. The results of the real-time PCR duplex examination of the blood sample were 28% positive, the results of the Epstein-Barr virus were 56% positive, and the results for both the Epstein-Barr virus and *T. gondii* were 16% positive. Regarding the cerebrospinal fluid samples, 20% were positive for *T. gondii*, 28% were positive for the Epstein-Barr virus, and 4% were positive for both the Epstein-Barr virus and *T. gondii*. A further study of quantitative PCR real-time for *T. gondii* and EBV should be conducted to enhance sensitivity (i.e., the detection threshold). In addition, for confirmation, further research should be conducted on blood samples alone to detect *T. gondii* and the Epstein-Barr virus.

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