The Use of PCR in Detecting Toxoplasma Parasites in the Blood and Brains of Mice Experimentally Infected with *Toxoplasma gondii*

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Abstract

Polymerase chain reaction (PCR) has been extensively used for diagnosis recently because of its very high sensitivity and specificity. We studied the applicability of PCR to the early diagnosis of toxoplasmosis in a murine model orally infected with *Toxoplasma gondii* (S-273). PCR was performed using EH24 and HE27 primers synthesized by the phosphoramidite method. Mice blood and brains collected on various post infection days (PID) were analysed by PCR (35 cycles). A portion of the brain tissue from each mouse was examined microscopically for the presence of parasite cysts. Blood and brain PCR were positive on the 9th and 12th day post-infection (DPI). Toxoplasma cysts in brain tissue appeared only on the 18th PID. The results showed that *Toxoplasma* parasites can be detected earlier in the blood than in the brain during primary infection, indicating that blood PCR is the more useful procedure.

Introduction

*Toxoplasma gondii* causes various types of clinical syndromes in man including blindness, congenital disorders, abortion and fatal toxoplasmic encephalitis (TE). Life-threatening infection occurs primarily among immunocompromised patients. Thus, *T. gondii* is one of the most common opportunistic pathogens that cause TE among patients with acquired immunodeficiency syndrome (AIDS)1)-3), organ transplant recipients4)-3) and others6)-7). An outbreak of toxoplasmic encephalitis has also been reported in Western Hemisphere which is attributed to the spread of HIV infection and AIDS8). TE in immunocompromised patients reportedly results from reactivation of latent infection9).

The diagnosis of toxoplasmosis in immunocompromised patients is problematic. Serological methods are not helpful because either no antibody is produced or it is delayed10). The isolation of parasites by tissue culture or mouse inoculation are either insensitive or time consuming. To overcome this problem, various investigators have used polymerase chain reaction (PCR) to diagnose toxoplasmosis in different clinical samples11)-16) using various primers13)-17). PCR is reportedly very sensitive and specific for the diagnosis of toxoplasmosis11)-17). We evaluated the use of PCR in...
detecting the Toxoplasma parasite in the blood and brains of experimentally infected mice on various post infection days (PID) along with the microscopic detection of parasitic cysts in brain tissues.

**Materials and Methods**

Mice and inoculation: Ten female ICR mice (Nippon CLEA Company, Japan) aged 4–5 weeks were infected with *T. gondii* (S-273) bradyzoites obtained from previously infected mice brains. Briefly, mice brains containing approximately 20 cysts/4 mg brain tissue were homogenized in 5 ml of minimum essential medium (MEM) and mice were fed with 0.1 ml of the brain homogenate. The infection day was designated as day 0.

Blood and brain samples: Blood and brain samples were obtained by sacrificing one infected mouse on PID 2, 4, 6, 9, 12, 15, 18, 21, 24 and 27. The mice were killed by ether inhalation and the abdomen was immediately exposed. Blood was collected from the abdominal aorta using a 1.0 ml syringe. About 1.0 ml blood was collected from each of the mice, of which 0.6 ml was mixed with ethylene diamine tetra-acetic acid (EDTA) anticoagulant and the remainder was transferred to Microtainers (45 × 8 mm) (Becton Dickinson, USA) for serum separation. Subsequently, the whole brain was removed by opening the skull.

DNA extraction: DNA from blood and brain tissue was extracted as follows. A portion of brain tissue measuring about 2 mm³ was placed in a micro test tube (Eppendorf, Germany) and minced using surgical blades. The minced tissue was washed in 300 µl of Tris-NaCl-MgCl₂ (TNM) buffer centrifuged at 550 g. Then washed twice in Tris-NaCl-EDTA (TNE) buffer. The tissue precipitate was resuspended in 400 µl, into which 3 µl of 20% SDS and 30 µl of proteinase K (10 mg/ml) were added. The mixture was agitated at room temperature until the tissue was completely digested. Sepa Gene Reagent III (700 µl) and 400 µl of Reagent IV (Sanko Junyaku Co. Ltd., Japan) were added to the digest and mixed vigorously followed by centrifugation at 13,835 g for 10 min at 4°C. The clear supernatant was transferred into a micro test tube and 50 µl Sepa Gene Reagent V and 500 µl isopropanol were added followed by gentle mixing. After centrifugation at 13,835 g, the DNA precipitate was washed twice in 500 µl of 75% ethanol. The DNA was dried at 65°C in thermal cycler (ASTEC Co., Japan) for 10 min, then dissolved in 100 µl of Tris-EDTA buffer heating at 70°C for 15 min.

Blood (500 µl) was mixed with 2.0 ml of haemolysin reagent (Tris-Sucrose-Triton X100) and washed three times in the same reagent. EDTA-saline (2.0 ml), 200 µl of 10% SDS and 100 µl of proteinase K were added to the precipitate and agitated until it was completely dissolved. Phenol-saturated Tris-EDTA buffer (2.0 ml) was added to this mixture, shaken vigorously, then separated by centrifugation at 13,835 g. The supernatant was mixed with 2.0 ml of phenol-chloroform-isoamyl alcohol for 15 min then clarified by centrifugation at 13,835 g.

Primer: The primers Guay et al.¹⁷ reported were 5'-GGCACAAACGAGCGCCACGC-3' (EH24) and 5'-TCATGAAGATCTGTTCATT-3’ (HE27), were synthesized by phosphoramidite method.

PCR: The PCR mixture (95 µl) (Distilled water 64.75 µl, PCR Reagent buffer 10 µl, MgCl₂ 10 µl, 10 mM dATP 2 µl, 10 mM dCTP 2 µl, 10 mM dGTP 2 µl, 10 mM dTTP 2 µl, primer EH24 1 µl, primer HE27 1 µl, and DNA Taq polymerase 0.25 µl) (Perkin Elmer Cetus, USA) and 5.0 µl of DNA extract were mixed and overlaid with a drop of mineral oil (Sigma Chemical Co., USA) and thirtyfive cycles amplification were performed in PCR thermocycler (PC-700 ASTEC Co., Japan) along with positive and negative controls.

PCR product: The PCR product was mixed with loading buffer (4 : 1) and resolved by electrophoresis (Advance Co., Ltd., Japan: Mupid OZZ1) in 2% ultra pure agar (Life Technologies Inc., USA) gel and Tris-Borate-EDTA (TBE) buffer at 100 V for 25 min along with molecular weight markers.

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(Toyobo Co., Ltd. Japan). The gel was then stained in ethidium bromide, visualised under UV light (Mini Transluminator NTM-10, ASTEC Co., Japan) and photographed using polaroid camera (DS-300 camera, FUNAKOSHI Co., Japan).

Results

Of the ten mice blood samples analysed by PCR, *Toxoplasma gondii* parasites were detected only in the blood from PID 9 and not before or thereafter (Fig. 1A). Brain tissue PCR revealed *Toxoplasma* parasites from PID 12 until PID 27 (Fig. 1B). Direct microscopic examination of brain tissue revealed *Toxoplasma* cysts only from PID 18 (Fig. 2) and thereafter. The size of the cysts varied from 15 to 35 μm. The parasite was detected in the blood by PCR three days earlier than in the brain tissue PCR and 9 days before cysts were microscopically detectable. *Toxoplasma* antibodies appeared from PID 12 and remained thereafter (Fig. 3).

![Fig. 1](image_url)

**Fig. 1** Agarose gel (2.0%) electrophoresis of nucleic acid PCR products from mice (A) blood samples and (B) brain samples collected at various PID with EH24 and HE27 primers. (A) MM: Molecular markers, 3 to 18: blood PCR and Pc: Positive control. (B) MM: Molecular markers, 3 to 18 brain tissue samples PCR and Pc: Positive control (497 bp).

![Fig. 2](image_url)

**Fig. 2** Mice brain wet preparation showing *Toxoplasma gondii* (S273) cysts (×20) in light microscopy.
Use of PCR in Toxoplasma Parasite Detection

In this study, we evaluated the use of PCR in detecting Toxoplasma parasite in blood and brain tissue of experimentally infected mice at various PID. The PCR findings were also compared with direct microscopic examination of brain tissue for Toxoplasma cysts. We detected the parasite in the blood earlier by PCR (on PID 9) than in brain tissue (on PID 12 and thereafter). PCR revealed that the blood however, was positive only on PID 9 and not thereafter. This finding correlated with the natural course of primary infection, where the parasite disseminates through bloodstream, causing transient parasitemia before they localize in various tissues. However, the appearance of blood parasitemia in primary infection differs among parasite strains and routes of infection. Weiss et al. 18 detected Toxoplasma parasites by Southern blot hybridization within five days of infection in mice peritoneally infected with the RH strain of T. gondii. Early detection of blood parasitemia in their study is attributed to high virulence of the RH strain compared with S-273, which is of moderate.

Compared with blood and brain tissue PCR positivity at PID 9 and 12, direct microscopic examination of brain tissue revealed Toxoplasma cysts only on PID 18 and thereafter. This appears to be due to the time taken by parasite to form a recognisable tissue cyst. These findings illustrate that blood PCR is more helpful in the early diagnosis of toxoplasmosis, as has been indicated by other investigators.16,19 particularly in AIDS patients, as parasitemia is a common feature of T. gondii infection during profound immunocompromise. Though not helpful in the diagnosis of toxoplasmosis in AIDS patients, we also tested the mice serum samples for the detection of Toxoplasma antibodies. Toxoplasma antibodies were evident on PID 12, the same day upon which brain tissue PCR was positive.

As many investigators15–17 have shown, our results also support the high sensitivity and specificity of PCR. Hitt & Filice20 detected as low as 1–10 Toxoplasma parasites by means of PCR. However, they found a significantly higher detection rate by mouse inoculation (62.0%) compared with that by gene amplification (37.0%) and cell culture (25.0%). Their findings are in contrast to many other reports showing the superiority of PCR over conventional diagnostic methods.15–17. PCR however, is not cost-effective and cannot be performed in small laboratories. Moreover, the detection
of parasite in tissues by PCR cannot help to discriminate between active and latent infection\(^\text{13}\). Additionally, brain biopsy for the diagnosis of TE is not only difficult, but is also associated with high morbidity. Therefore, detection of \textit{Toxoplasma} parasites in blood appears to be useful in both early diagnosis and in discriminating between active and latent infections, as parasitemia commonly occurs as result of either primary or reactivated\(^\text{9}\) infection.

References
PCR 法を用いたトキソプラズマ感染実験マウスの血液および脳組織からのトキソプラズマ原虫の検出

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要 旨
ポリメラーゼ連鎖反応（PCR 法）は、高感度で特異性が高いことから近年臨床診断に広く用いられるようになった。筆者らはトキソプラズマ感染症早期診断における PCR 法の有用性を検討する目的で Toxoplasma gondii (S-273株) 経口感染マウスをモデルとした検討を行った。PCR 法にはEH24およびHE27プライマーを用いそれぞれホスホアミダイド法で合成した。マウスの血液と脳は被感染時より経時的に採取し、35サイクルのPCR 反応を実施した。また、各マウス脳組織の一部はトキソプラズマ原虫シストの存在を見るために顕微鏡下に観察した。血液と脳の PCR 法ではそれぞれ感染9日後と12日後陽性となった。脳組織中のトキソプラズマシストは感染18日後に検出された。以上の成績から、初感染においてトキソプラズマ原虫は脳よりも血液において早く検出され、血液を用いた PCR 法はより有用な方法であることが示された。