Article available at http://www.parasite-journal.org or http://dx.doi.org/10.1051/parasite/1995022181

DETECTION OF *TOXOPLASMA GONDII* PARASITEMIA BY POLYMERASE CHAIN REACTION IN PERORALLY INFECTED MICE

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Summary :

Sequential blood samples collected from mice infected perorally with an avirulent strain of *T. gondii* were analysed for parasite DNA by a polymerase chain reaction method (PCR). Two pairs of primers specific for gene B1 and the repetitive DNA sequence $TGR1_E$ were used for DNA amplification. Amplified products were detected by means of electrophoresis with ethidium bromide staining. Parasitemia was also determined by cell culture.
Parasitemia was never detected by the tissue culture method, whereas parasite DNA was continously detected with PCR from day 2 to day 21. These results confirm the high sensitivity of PCR for *T. gondii* DNA in blood, and show that circulating DNA is present for long periods in mice following primary infection.

KEY WORDS : toxoplasmosis. *Toxoplasma gondii*. PCR. tissue culture. parasitemia.
MOTS CLES : *toxoplasmose*. Toxoplasma gondii. *PCR. culture cellulaire. parasitémie.*

Résumé : DÉTECTION DE *TOXOPLASMA GONDII* PAR "POLYMERASE CHAIN REACTION" (PCR) DANS LE SANG DE SOURIS INFECTÉES PER OS.

Des prélèvements sanguins de souris infectées per os par une souche chronique de T. gondii *ont été séquentiellement analysés par PCR pour la détection d'ADN toxoplasmique. Deux couples d'amorces, l'un spécifique du gène B1, l'autre de la séquence répétée $TGR1_E$ ont été utilisés pour l'amplification génomique. Les produits d'amplification ont été révélés par électrophorèse sur gel coloré au bromure d'éthidium. Parallèlement la parasitémie était recherchée par culture cellulaire. Dans aucun des cas une parasitémie n'a pu être mise en évidence par culture alors que la PCR a permis de mettre en évidence du DNA toxoplasmique du 2e jour au 21e jour qui ont suivi l'infection. Ces résultats confirment la grande sensibilité de la PCR pour détecter la circulation sanguine d'ADN toxoplasmique et mettent en évidence le caractère prolongé de la durée de cette circulation chez la souris expérimentalement infectée.*

INTRODUCTION

In 1910, Nicolle and Manceau discovered *Toxoplasma gondii* by microscopic examination of a blood smear from a rodent (*Ctenodoctylus gundi*). However, direct observation of the parasite in blood is rare, and parasitemia can usually be only demonstrated by subinoculation of blood to mice or cell lines. More recently, several studies have shown the sensitivity of the polymerase chain reaction (PCR) for toxoplasmic DNA in various biological specimens (aqueous humor; heart; brain and liver biopsy; cebrospinal fluid; bronchoalveolar lavage fluid; amniotic fluid) and blood (Johnson *et al.*, 1990; Ho-Yen *et al.*, 1992; Filice *et al.*, 1993; Dupouy-Camet *et al.*, 1993). However, little is known of the kinetics of parasitemia following primary infection.

We used a PCR technique to detect *T. gondii* DNA in blood from mice infected perorally with an avirulent strain.

MATERIALS AND METHODS

TOXOPLASMA STRAIN AND INFECTION OF MICE

The avirulent C strain of *T. gondii* was used. It was initially isolated from an infected human placenta (Beauvais *et al.*, 1982) and is maintained in mice by 6-monthly passage by intraperitoneal inoculation of 25 brain cysts. Sixty mice (Swiss Webster females, weighing 20-24g, IffaCredo, France) were infected by gavage with 0.2 ml of a brain homogenate in saline, containing 20 cysts.

Blood samples were collected from the orbital venous sinus into EDTA anticoagulated tubes. Five mice were killed 12, 24, 36 and 48 hours and 4, 7, 11, 21 and 30 days post infection. Blood samples from five mice were pooled for detection of *T. gondii* by PCR and cell culture.

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Primers used for detection of B1 gene
5' CCGCC TCC T TCG TCCG TCG TA
5' TGAAGAGAGGAAACAGGTGGTCG

Primers used for detection of $TGR1_E$ DNA sequence
5' ATGGTCCGGCCGGTGTATGATATGCGAT
5' TCCC TACGTGGTGCCGCAGTTGCCT

Fig. 1. – Synthetic oligonucleotide primers

Polymerase chain reaction

Each pooled blood sample was mixed with an equal volume of sterile 2% Dextran T 500 (Pharmacia) in normal saline. After 15 min of bench-top sedimentation, the erythrocyte-free supernatant containing leukocytes and platelets was collected. After centrifugation at 2000 x g for 10 min, the pellet was washed twice in sterile phosphate-buffered saline (PBS) and kept frozen at -20°C.

PCR was based on the detection of gene B1 (Burg *et al.,* 1988) and the repeated sequence $TGR1_E$ (Cristina *et al.,* 1991); synthetic oligonucleotide primers (Fig. 1) were supplied by Genset (Paris). DNA was extracted from the frozen cell pellets with phenol (Hermann *et al.,* 1987). Strict measures to reduce contamination were used (Kwok *et al.,* 1989) : the four steps of the reaction (DNA extraction, reagent preparation, amplification and analysis) were carried out in four differents rooms. We used the GeneAmp® PCR carry-over prevention kit (Perkin Elmer Cetus) to avoid false-positive amplification by the substitution of dUTP for dTTP in the PCR reaction mix and treatment of subsequent PCR amplification products with uracil N-glycosylase (UNG) (Longo *et al.,* 1990).

The amplification reactions were induced with Taq DNA polymerase (Cetus) in a Perkin-Elmer Cetus thermal cycler. Briefly, 1.25 U of Taq polymerase was added to a 50 ml reaction volume containing 40 ml of reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM MgCl2, 0.01% gelatin), 500 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dUTP), 0.5 mM oligonucleotides, 0.5 U of UNG and 10 ml of purified DNA. Mineral oil (50 ml) was added to each reaction mix to prevent evaporation. An initial incubation at 50°C for 5 min allowed UNG to destroy any amplified product containing deoxyuridine triphosphate that could have been carried over from previous reactions. Then, after an initial cycle consisting of 5 min of denaturation at 94°C, 2 min of annealing at 65°C and 40 s of extension at 72°C, the following cycles were repeated 35 times : 30 s of denaturation at 94°C, 40 s of annealing at 65°C and 40 s of extension at 72°C. The final step consisted of 30 s of denaturation at 94°C, 40 s of annealing at 65°C, and 5 min of extension at 72°C. After the final PCR reactions, the temperature was held at 72°C and 50 ml of choroform was added to each tube to prevent degradation of PCR products by residual or reactivated UNG. The amplification products (10 ml) were stained with ethidium bromide and submitted to electrophoretic migration in 3% agarose gels. Positive controls (DNA extracted from the RH strain of *T. gondii*) and negative controls (distilled water) were used in each set of experiments and results were considered valid only if control results were as expected.

Cell culture

Each blood sample was inoculated into MRC5 cell cultures prepared in 96-well tissue culture plates (Derouin *et al.,* 1991). Briefly, 200ml of blood was suspended in 4 ml of PBS, 0.15 M NaCl, pH 7.2, supplemented with penicillin 100 U/ml, streptomycin 100 mg/l and amphotericin B 5 mg/l. Serial fourfold dilutions were prepared in minimal essential medium (MEM) supplemented with glutamine 290 mg/l, penicillin 50 iu/ml, streptomycin 50 mg/l and heat-inactivated fetal calf serum (Flow Laboratories, Paris, France). Forty microliters of each dilution was inoculated into duplicate wells of 96-well tissue culture plates and incubated for 72 hours at 37°C. Cultures were examined for Toxoplasma growth in an indirect immunofluorescence assay.

RESULTS AND DISCUSSION

Mice showed no physical signs of infection. The results of PCR and cell culture are presented in table I. Tissue cultures were always negative, whereas PCR was positive as early as 48 hours post-infection. PCR remained positive on days 4 and 7 with B1 gene amplification, and on days 4, 7, 11, 14 ,21 with TGR1E sequence amplification.

The results obtained by tissue culture were in agreement with our previous findings using the same strain of *T. gondii*, in which mice were infected intraperitoneally or perorally and tested from 12 h to 90 days after infection. Parasitemia was positive only once, 14 days after intraperitoneal inoculation (Derouin *et al.,* 1991) and never after oral infection (Sumuyen *et al.,* 1995).

Both PCR gene amplifications were positive until day 7, but only the TGR1E sequence was positive after this date. This difference in the results obtained with the two targets can be attributed to the probable higher repetition of the TGR1E sequence (unknown) compared to the B1 gene (repeated 35-fold). In preliminary experiments with RH strain tachyzoite DNA

		Time post-infection*										
		0	12h	24h	36h	48h	D4	D7	D11	D14	D21	D30
PCR	B_1	-	-	-	-	+	+	+	-	-	-	-
	TGR_1E	-	-	-	-	+	+	+	+	+	+	-
Cell culture	-	-	-	-	-	-	-	-	-	-	-	-

Table I. – Detection of Toxoplasma (Coul strain) by PCR (electrophoresis and ethidium bromide staining) and cell culture in the blood of perorally infected mice.
* Each sample was a pool of blood from 5 mice tested in parallel by PCR and cell culture.

diluted in water, serial dilutions were amplified. We detected approximately a DNA dilution corresponding to 0.1 organism with B1 and 0.01 with TGR_1E. Wastling *et al.,* 1993 compared B1 and P30 (a single-copy gene) detection by PCR in sheep inoculated subcutaneously : B1 PCR was consistently more sensitive than P30 PCR, being positive on peripheral blood earlier and for significantly longer days (6 to 11 after infection, compared to days 7 and 8).

Like other reports, our results indicate that parasite DNA circulation in experimentally infected animals is not transient. Joss *et al.,* 1993 detected toxoplasma DNA (Beverley strain) in blood from Cotton rats and mice by means of B1 PCR from days 2 to 32 after intraperitoneal inoculation. Hitt *et al.,* 1992 detected toxoplasma DNA (C 56 strain) in blood by B1 PCR from days 6 to 32 after subcutaneous inoculation of rabbits. Schoondermark et al 1993 detected toxoplasma DNA (RH strain) in blood of pregnant monkeys by means of ribosomal PCR for about 10 days after intravenous inoculation. Miedouge 1994 (personal communication) detected toxoplasma (Beverley strain) in blood 7 to 28 days after intraperitoneal inoculation of mice.

No data on the kinetics of circulating DNA during naturally acquired infection are available. Experimental studies of parasitemia in chronic infection by avirulent strains (Regminton *et al.,* 1961; Dubey *et al.,* 1980) or acute infection with virulent strains (Ruchman *et al.,* 1951, Jacobs *et al.,* 1950 and 1953) have been based on subinoculation of blood; parasitemia was inconsistently detected with avirulent strains contrary to the virulent strains.

Our results show that durable parasitemia can be detected by PCR after infection of mice with an avirulent strain. Parasitemia was detected by PCR until day 21, but not at day 30, when the immune response probably becomes effective. These results indicate that PCR could be useful for very early diagnosis of toxoplasmosis, before the emergence of specific antibodies.

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Accepté le 16 février 1995