Comparison of PCR, capture ELISA and immunoblotting for detection of *Toxoplasma gondii* in infected mice

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PCR was compared with capture ELISA and immunoblotting for the detection of *Toxoplasma gondii* in sera of acutely infected mice. One hundred animals were inoculated intraperitoneally with 5000 trophozoites of RH strain and five of them were killed every 3 h from 3 h to 21 h post infection (p.i.), and every day from day 1 to day 7 p.i.. No assay detected the parasite from 3 h p.i. to 15 h p.i. PCR was the most sensitive assay and detected the *T. gondii* from 18 h p.i., whereas the other assays detected it only from day 1.

**Introduction**

*T. gondii* is an intracellular protozoan parasite that is able to infect all warm-blooded animals and causes toxoplasmosis. This disease is generally benign and has little clinical relevance in immunocompetent subjects. However, toxoplasmosis can be serious or fatal in children infected during their intra-uterine development and in immunocompromised patients in the absence of early diagnosis and a suitable treatment. The classical diagnosis of toxoplasmosis relies on qualitative or quantitative detection of specific antibodies, or both. However, serological methods have poor efficiency, especially in neonates and in immunodeficient patients. To improve this diagnosis, there have been many studies with various immunological assays to detect the parasite or its components either in experimental infection [1, 2] or in human toxoplasmosis [3, 4]. Recently, PCR assays for the detection of *T. gondii* DNA were developed and primers specific to different regions of the genome of the parasite have been used [5, 6]. Several authors have evaluated this method for the diagnosis of different forms of toxoplasmosis in various human samples and have demonstrated the high sensitivity and specificity of PCR performed on amniotic fluids for the diagnosis of congenital toxoplasmosis [7–12]. In experimentally infected animals, PCR assay for the *B1* gene has been performed on whole blood and compared with cell culture and mouse inoculation. Several reports showed that PCR was more sensitive than cell culture but less sensitive than mouse inoculation [13–16]. The present study evaluated and compared the PCR assay with two immunological techniques (capture ELISA and immunoblotting) for the detection of *T. gondii* in serum specimens of mice acutely infected with the RH strain of *T. gondii*. The purpose of early detection of *T. gondii* in experimentally infected mice is to improve the diagnosis of toxoplasmosis in human adults and neonates.

**Materials and methods**

**Mice sera**

Female OF-1 mice weighing 18–22 g were infected by the intraperitoneal route with $5 \times 10^7$ trophozoites of *T. gondii* RH strain prepared as described previously [17]. Five animals were killed at 3, 6, 9, 12, 15, 18 and 21 h and 1, 2, 3, 4, 5, 6 and 7 days post-infection (p.i.), respectively. Whole blood was removed from each animal by jugular venepuncture, and centrifuged for 10 min at 200 g. Sera were kept frozen at $-20^\circ$C until use. Sera from five uninfected mice were used as negative controls.

**Capture ELISA**

Capture ELISA was performed as described previously [2]. Briefly, the wells of microtiteration plates (EIA,
Costar, Cambridge, MA, USA) were coated overnight at 4°C with 100 μl of the polyclonal antiserum to T. gondii diluted in carbonate buffer, pH 9.6, to contain 5 μl of protein/ml. After saturation with bovine serum albumin (BSA) 1% and washing, 100 μl of serum samples diluted 1 in 8 in PBS containing BSA 1% and Tween 20 0.05% (PBT) were added to each well and held for 1 h at 37°C. After washing, 100 μl of the same polyclonal antiserum labelled with alkaline phosphatase and diluted 1 in 500 in PBT were added to each well and incubated for 1 h at 37°C. After washing and addition of the substrate (p-nitrophenylphosphate; Sigma), the reaction was stopped. The threshold optical density at 405 nm (OD405) value separating positive and negative reactions was established at 0.137 (mean value of 20 uninfected mouse serum specimens plus 10 SD). Each sample was tested in duplicate and the results are expressed as the arithmetic mean of OD405.

**Immunoblotting**

Mouse serum samples were diluted 1 in 50 in 0.5 M Tris-HCl buffer containing 2-mercaptoethanol 5% and sodium dodecyl sulphate 2%. Electrophoresis, electrotransfer and immune detection were performed as described previously [2]. Briefly, the optical density of samples was resolved on polyacrylamide 12% gels and blotted onto polyvinylidene difluoride membranes (Immobilon®; Millipore, Bedford, MA, USA). After saturation of the blots with 0.01 M Tris-saline buffer containing skimmed milk 5%, antigens were revealed by two-step immuno-enzymic reaction with a polyclonal rabbit antiserum diluted 1 in 100 and peroxo-

idase-conjugated swine anti-rabbit serum (Dako) diluted 1 in 300. The substrate consisted of a solution of O-dianisidine 0.01% and H2O2 0.003%. The molecular masses of proteins specific to T. gondii (22–110 kDa) were determined by reference to commercially available standards (BioRad Laboratories, Richmond, CA, USA). Immunoblotting was performed twice.

**PCR assay**

To determine the sensitivity of the PCR kit, free trophozoites of T. gondii RH strain obtained from a mouse ascites fluid were washed three times in saline solution (NaCl 0.9%) by centrifugation at 200 g. After enumeration by microscopy, 10-fold dilutions were done in distilled water, corresponding to 10–

10 trophozoites/ml.

The DNA extraction was performed on 200 μl of serum from mice (infected and non-infected) and each trophozoite dilution with a high pure PCR template preparation kit (Boehringer Mannheim, Meylan, France) in accordance with the manufacturer’s instruc-

tions. Briefly, 200 μl of sample were added to 200 μl of binding buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, Triton X-100 20%, pH 4.4) and 40 μl of proteinase K. After incubation at 72°C for 10 min, 100 μl of isopropanol were added and the samples were centrifuged for 1 min at 6000 g. DNA was specifically bound to silica membranes in the presence of a chaotropic salt. After two washes with an appropriate buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5), nucleic acid was eluted with 50 μl of the elution buffer (10 mM Tris, pH 8.5).

Amplification of the B1 gene was performed according to the method of Burg et al. [6] with two primers: 10 μl of DNA product extraction were added to 40 μl of reaction mixture containing 10× PCR buffer (200 mM Tris-HCl, 500 mM KCl, pH 8.4; Gibco BRL, Eragny, France) 5 μl, 50 mM MgCl2 (Gibco BRL) 3 μl, 0.2 mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dUTP; Boehringer Mannheim), 25 pmol of each primer (5’-AACGCGAGTAGCACCTGAGGAGA-3’ and 5’-TGGTCTACGTCGA TGCGCATGACAC-3’; Eurogentec), Taq-DNA poly-

merase (Gibco BRL) 2.5 IU and sterile water. After the addition of 50 μl of mineral oil to prevent evaporation, the reaction was performed in a Thermocolis thermo-

cycler (Cis Bio International, Gif sur Yvette, France). After an initial denaturation step at 95°C for 5 min, 40 cycles were run, consisting of denaturation (95°C for 1 min), annealing (58°C for 45 s), extension (72°C for 45 s) and a final extension at 72°C for 10 min. To prevent potential carry-over contamination of the amplified target DNA from previous reactions, the PCR reaction mix was initially incubated with uracil N-glycosylase for 5 min at 50°C according to the method of Longo et al. [18].

PCR products (12 μl) and a DNA mol. wt marker (50-

bp ladder, Boehringer Mannheim) were analysed simultaneously by electrophoresis in an agarose 3% w/v gel (Metaphor® Agarose, Tebu) in 1× TAE buffer (0.04 M Tris, 0.001 M EDTA; Bioprobe) and amplified fragments of 115 bp were visualised under UV illumination after staining with ethidium bromide (Sigma) for 10 min.

To avoid contamination, the DNA extraction, prepara-

tion of mixture reaction, amplification and revelation steps were performed in separate rooms. A positive control (DNA extracted from the 105 trophozoites/ml concentration) and two negative controls (mixture reaction and DNA extracted from non-infected mice) were tested in each experiment. Control and assay samples were tested in duplicate.

**Results**

In this study capture ELISA, immunoblotting and PCR assays were performed in duplicate and the same results were obtained in each reaction of the respective assay. All sera taken from non-infected mice gave negative results by the three assays.
PCR assay allowed the detection of two trophozoites in 200 µl, corresponding to a sensitivity of c. 0.4 pg of DNA, as one trophozoite contains c. 0.2 pg of DNA [5]. When a similar experiment was performed with trophozoites diluted in non-infected mouse serum specimens, the same sensitivity was observed.

The comparison of results obtained by PCR with those obtained by capture ELISA and immunoblotting is presented in Table 1. The three methods used did not detect the presence of the parasite from 3 h to 15 h p.i. However, PCR was more sensitive than both immunological assays (p < 0.01); it was weakly positive in three of five animals killed at 18 h p.i. and was positive in all animals killed at 21 h and on days 1–7 p.i. Fig. 1a shows results obtained at day 5 p.i.

By capture ELISA the presence of the parasite was detected in all animals from day 1 p.i. and the OD405 increased moderately up to day 4 p.i. (values were 0.176, 0.170, 0.184 and 0.232 for days 1, 2, 3 and 4, respectively), and strongly for days 5, 6 and 7 (values were 0.377, 0.451 and 0.421, respectively).

By immunoblotting (Fig. 1b), three polypeptides with mol. wts of 110, 87 and 75 kDa were detected in all animals on days 1–7 p.i. From day 5, four more polypeptides of lower mol. wt appeared: 48, 30, 24 and 22 kDa.

**Discussion**

The aim of the present study was to compare PCR with capture ELISA and immunoblotting for the detection of *T. gondii* in serum specimens of mice infected intraperitoneally with the RH strain. The originality of the study relies on the follow-up of the parasitaemia in acute experimental toxoplasmosis by using an inoculum that allowed survival of the animals for as long as

<table>
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<tr>
<th>Time post-infection</th>
<th>Number of sera tested</th>
<th>Number of positive tests obtained by</th>
<th>Capture ELISA</th>
<th>Immunoblotting</th>
<th>PCR</th>
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<td>3, 6, 9, 12 and 15 h</td>
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<td>21 h</td>
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<td>Days 1–7</td>
<td>35</td>
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Five animals were killed at each time point.

*p < 0.01 (χ² test).

**Fig. 1.** Detection of *T. gondii* RH strain by PCR (a) and immunoblotting (b) in sera of mice infected with 5000 trophozoites. D, day post-infection; Ag, antigenic extract of *T. gondii*; M, mol. wt marker (50-bp ladder); T⁻, negative control; T⁺, positive control (DNA extracted from the 10⁴ trophozoites/ml concentration). Arrows indicate *T. gondii* proteins (22–110 kDa).
DETECTION OF T. GONDII BY PCR IMMUNOBLOTTING AND CAPTURE ELISA

possible (7 days). The simultaneous detection of T. gondii proteins by immunological techniques and of DNA by PCR suggests that the parasite or its components are present in blood at an early stage of infection and persist until the death of the animals, despite the appearance of antibodies at day 4 p.i. [17].

The sensitivity of the PCR method in this study was c. two trophozoites. These findings are in agreement with those of Hitt and Filice [13] (sensitivity between 0.5 and 5 trophozoites) and of El Sayed Khalifa et al. [19] (1 trophozoite); however, the latter authors reported a decrease in sensitivity to c. 10 trophozoites in the presence of leucocyte DNA. The results of the present study suggest that the PCR is more sensitive than immunological methods. The first positive result was found by PCR at 18 h versus 24 h p.i. by capture ELISA and immunoblotting. Moreover, the B1 gene of T. gondii was amplified from serum specimens instead of the whole blood or leucocyte preparations described in other studies, with similar sensitivity and rapidity.

The results of the present study are singular because no other study has been performed to compare PCR with immunological techniques, rather than comparison with cell culture and mouse inoculation techniques. Paugam et al. [14] detected DNA on days 2–21 p.i. in whole blood of mice perorally infected with cysts of the C strain by two PCR tests with primers specific to the B1 gene and the TGR I region, respectively, whereas all cell culture results were negative. In an acute murine model with the RH strain described by Weiss et al. [20], the P30 gene of T. gondii was detected by PCR in the tissue by day 2 p.i. and in the blood of animals by day 5 p.i. In another study [21], the toxoplasma B1 gene was detected by PCR at five stages between 16 and 66 h p.i. in leucocyte preparations from mice and rats infected with trophozoites of the RH strain, and at 12 stages between 2 and 38 days p.i. when those animals were inoculated by cysts of the Beverley strain. In the latter case, PCR was more sensitive than animal inoculation. However, in purified leucocytes from a rabbit subcutaneously infected with 10^7 tachyzoites of the C56 strain, the positivity of the B1 gene PCR amplification was obtained later (6–32 days p.i.) [13]: tests for parasitaemia became positive in 62% of infected samples by mouse inoculation, 37% by PCR and 25% by cell culture assay. In another animal model described by Wastling et al. [22], two PCR tests with primers specific to the B1 and P30 genes were compared with mouse inoculation for assays on effrent lymph and peripheral blood collected from sheep infected subcutaneously with 10^7 tachyzoites of T. gondii S48 strain. The amplification of the B1 gene was consistently more sensitive than that of the P30 gene and the results agreed closely with those from mouse inoculation. In the detection of T. gondii in a range of tissues in sheep and cattle challenged with different doses of sporulated oocysts, the PCR assay was much more sensitive and specific than histological assay [16, 23]. This greater sensitivity of the PCR has been confirmed in the diagnosis of congenital toxoplasmosis by many others [24, 25].

Despite their heterogeneity, all these reports indicate that B1 gene PCR (whatever B1 gene sequence is amplified) is more sensitive and rapid than capture ELISA, immunoblotting or cell culture assays. However, the sensitivity of the results depends on the strain of T. gondii, the load of the inoculum and the PCR protocols used.

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