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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 5×10^3 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°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 microtitration plates (EIA,

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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, electro-transfer and immune detection were performed as described previously [2]. Briefly, the proteins of samples were separated on polyacrylamide 12% gels and blotted on to polyvinylidene difluoride membranes (Immobilon^R; 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 peroxidase-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 10⁷–10⁻¹ 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 instructions. 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 R 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 *BI* 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'-AACGGGCGAGTAG CACCTGAGGAGA-3' and 5'-TGGGTCTACGTCGA TGGCATGACAAC-3'; Eurogentec), Taq-DNA polymerase (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 Thermocis thermocycler (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 uracyl 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^R 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, preparation of mixture reaction, amplification and revelation steps were performed in separate rooms. A positive control (DNA extracted from the 10⁷ 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 μ l, 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 intra-peritoneally 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 1. Comparison of results obtained by capture ELISA, immunoblotting and PCR in the detection of *T. gondii* RH strain in sera of mice infected with 5000 trophozoites

Time post-infection	Number of sera tested	Number of positive tests obtained by		
		Capture ELISA*	Immunoblotting*	PCR*
3, 6, 9, 12 and 15 h	25	0	0	0
18 h	5	0	0	3
21 h	5	0	0	5
Days 1–7	35	35	35	35

Five animals were killed at each time point.

* $p < 0.01$ (χ^2 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 10⁷ trophozoites/ml concentration). Arrows indicate *T. gondii* proteins (22–110 kDa).

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_E* 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 Beverly strain. In the latter case, PCR was more sensitive than animal inoculation. However, in purified leucocytes from a rabbit subcutaneously infected with 10⁴ 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 efferent lymph and peripheral blood collected from sheep infected subcutaneously with 10⁵ 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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References

1. Raizman RE, Neva FA. Detection of circulating antigen in acute experimental infections with *Toxoplasma gondii*. *J Infect Dis* 1975; **132**: 44–48.
2. Hafid J, Tran Manh Sung R, Pozzetto B *et al.* Kinetic of circulating antigens by capture ELISA and immunoblotting in murine toxoplasmosis. *Eur J Protistol* 1991; **27**: 40–45.
3. Van Knapen F, Panggabean SO. Detection of circulating antigen during acute infections with *Toxoplasma gondii* by enzyme-linked immunosorbent assay. *J Clin Microbiol* 1977; **6**: 545–547.
4. Hafid J, Tran Manh Sung R, Raberin H, Akono ZY, Pozzetto B, Jana M. Detection of circulating antigens of *Toxoplasma gondii* in human infection. *Am J Trop Med Hyg* 1995; **52**: 336–339.
5. Savva D. Isolation of a potential DNA probe for *Toxoplasma gondii*. *Microbios* 1989; **58**: 165–172.
6. Burg JL, Grover CM, Pouletty P, Boothroyd JC. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol* 1989; **27**: 1787–1792.
7. Grover CM, Thulliez P, Remington JS, Boothroyd JC. Rapid prenatal diagnosis of congenital *Toxoplasma* infection by using polymerase chain reaction and amniotic fluid. *J Clin Microbiol* 1990; **28**: 2297–2301.
8. Johnson JD, Butcher PD, Savva D, Holliman RE. Application of the polymerase chain reaction to the diagnosis of human toxoplasmosis. *J Infect* 1993; **26**: 147–158.
9. Bretagne S, Costa J-M, Fleury-Feith J, Poron F, Dubreuil-Lemaire M-L, Vidaud M. Quantitative competitive PCR with bronchoalveolar lavage fluid for diagnosis of toxoplasmosis in AIDS patients. *J Clin Microbiol* 1995; **33**: 1662–1664.
10. Fuentes I, Rodriguez M, Domingo CJ, del Castillo F, Juncosa T, Alvar J. Urine sample used for congenital toxoplasmosis diagnosis by PCR. *J Clin Microbiol* 1996; **34**: 2368–2371.
11. Cazenave J, Forestier F, Bessieres MH, Broussin B, Begueret J. Contribution of a new PCR assay to the prenatal diagnosis of congenital toxoplasmosis. *Prenat Diagn* 1992; **12**: 119–127.
12. Hohlfield P, Daffos F, Costa J-M, Thulliez P, Forestier F, Vidaud M. Prenatal diagnosis of congenital toxoplasmosis with a polymerase-chain-reaction test on amniotic fluid. *N Engl J Med* 1994; **331**: 695–699.
13. Hitt JA, Filice GA. Detection of *Toxoplasma gondii* parasitemia by gene amplification, cell culture, and mouse inoculation. *J Clin Microbiol* 1992; **30**: 3181–3184.
14. Paugam A, Dupouy-Camet J, Sumuyen M-H, Romand S, Lamoril J, Derouin F. Detection of *Toxoplasma gondii* parasitemia by polymerase chain reaction in perorally infected mice. *Parasite* 1995; **2**: 181–184.
15. Owen MR, Trees AJ. Vertical transmission of *Toxoplasma gondii* from chronically infected house (*Mus musculus*) and field (*Apodemus sylvaticus*) mice determined by polymerase chain reaction. *Parasitology* 1998; **116**: 299–304.
16. Esteban-Redondo I, Maley SW, Thomson K *et al.* Detection of *T. gondii* in tissues of sheep and cattle following oral infection. *Vet Parasitol* 1999; **86**: 155–171.
17. Hafid J, Tran Manh Sung R, Raberin H. Détection des antigènes circulants de *Toxoplasma gondii* par réaction

- d'immunoprécipitation dans la toxoplasmose murine. [Detection of circulating antigens of *Toxoplasma gondii* using immunoprecipitation reactions in murine toxoplasmosis.] *Ann Soc Belg Med Trop* 1989; **69**: 49–56.
18. Longo MC, Berminger MS, Hartley JL. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 1990; **93**: 125–128.
 19. El Sayed, Khalifa K, Roth A, Roth B, Arasteh KN, Janitschke K. Value of PCR for evaluating occurrence of parasitemia in immunocompromised patients with cerebral and extracerebral toxoplasmosis. *J Clin Microbiol* 1994; **32**: 2813–2819.
 20. Weiss LM, Udem SA, Salgo M, Tanowitz HB, Wittner M. Sensitive and specific detection of toxoplasma DNA in an experimental murine model: use of *Toxoplasma gondii*-specific cDNA and the polymerase chain reaction. *J Infect Dis* 1991; **163**: 180–186.
 21. Joss AWL, Chatterton JMW, Evans R, Ho-Yen DO. *Toxoplasma* polymerase chain reaction on experimental blood samples. *J Med Microbiol* 1993; **38**: 38–43.
 22. Wastling JM, Nicoll S, Buxton D. Comparison of two gene amplification methods for the detection of *Toxoplasma gondii* in experimentally infected sheep. *J Med Microbiol* 1993; **38**: 360–365.
 23. Esteban-Redondo I, Innes EA. Detection of *Toxoplasma gondii* in tissues of sheep orally challenged with different doses of oocysts. *Int J Parasitol* 1998; **28**: 1459–1466.
 24. Foulon W, Pinon J-M, Stray-Pedersen B *et al.* Prenatal diagnosis of congenital toxoplasmosis: a multicenter evaluation of different diagnostic parameters. *Am J Obstet Gynecol* 1999; **181**: 843–847.
 25. Fricker-Hidalgo H, Pelloux H, Racinet C *et al.* Detection of *Toxoplasma gondii* in 94 placentae from infected women by polymerase chain reaction, in vivo and in vitro cultures. *Placenta* 1998; **19**: 545–549.