Toxoplasma polymerase chain reaction on experimental blood samples

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Summary. A two-stage polymerase chain reaction (PCR) assay employing oligonucleotide primers from the B1 gene of *Toxoplasma gondii* was developed and assessed for sensitivity and specificity. It was able to detect *T. gondii* DNA from as little as one parasite/sample in mock-infected rat or mouse leucocyte preparations. Parasitaemia was also identified in animals at five stages between 16 and 66 h after infection with the virulent R_H strain, and at 12 stages between 2 and 38 days after infection with the cyst-forming Beverley strain. In the latter case, PCR was more sensitive than animal culture. No cross-reactions were observed in samples containing various opportunist pathogens which may also be found in the blood of immunocompromised patients.

Introduction

Laboratory diagnosis of human toxoplasmosis has relied traditionally on serological tests. This approach was satisfactory when most infections were primary infections in otherwise healthy patients, but now that tests for infection in immunocompromised patients are required, traditional serological tests are inadequate.^{1,2} Patients often do not mount an antibody response which can distinguish current from past infection; specific IgM is frequently not detectable nor is the total antibody elevated.^{1,2} In addition, fetal or congenital infection cannot always be diagnosed by serology alone; demonstration of *Toxoplasma gondii* by animal inoculation of blood or amniotic fluid is essential to maximise sensitivity.^{3,4}

The polymerase chain reaction (PCR) promises to be the most sensitive rapid means of demonstrating microbial DNA in clinical specimens.^{5,6} The presence of *T. gondii* in brain tissue from two patients with toxoplasma encephalitis was demonstrated by PCR primed with oligonucleotides from the P30 gene.⁷ The parasite can also be demonstrated in simulated infected CSF samples with primers from the B1 gene.⁸ For routine diagnostic purposes, blood samples are more convenient; however, toxoplasma antigen has been demonstrated in such samples with varying degrees of sensitivity and specificity.^{9–11} Toxoplasma DNA was detected with B1 gene primers in the presence of 10⁵ white blood cells, a concentration which might be seen in inflamed CSF samples.⁸ In contrast, a positive result was found in only one of 12 infected mouse blood samples tested with the P30 PCR.⁶ The current study examined the suitability of

blood as a specimen source for PCR tests on samples from experimentally infected rodents in a B1 oligonucleotide primed assay.

Materials and methods

Samples

Axillary vein blood samples were collected in lithium heparin and centrifuged on Histopaque (Sigma) (five volumes of sample on four volumes of Histopaque, at 1250 g 12.5 min). A broad interphase fraction containing the leucocytes was washed a further three times with sterile phosphate-buffered saline (PBS) pH 7.4. The final pellets were resuspended in PBS for animal inoculation. PCR samples were given one final wash in water to remove haemoglobin, before resuspension in water and storage at –70 °C.

T. gondii tachyzoites from Cotton rat peritoneal passage were washed three times with saline and resuspended at an appropriate concentration. Adenovirus, herpes simplex virus, cytomegalovirus, *Staphylococcus aureus* and *Candida albicans* were deposited by centrifugation from culture fluid, washed twice in PBS and resuspended at an appropriate concentration. They were reconstituted for testing at concentrations higher than necessary to prove infection—up to at least five-fold the concentration of each virus required to infect a tissue culture tube, 40–2000 cfu/test of *S. aureus* and 50–5000 cfu/test of *C. albicans*. *Pneumocystis carinii* cysts were washed from a piece of infected human lung and diluted from a stock containing 5 × 10⁶ cysts/ml, to working concentrations of 40–1000 cysts/test.

Samples in water, either fresh or previously frozen,

Fig. 1. Sensitivity of primary and nested PCR. Electrophoresis results following primary and nested PCR with primers S114+S115, then S114+S331, respectively. Lanes 1 and 14, 123-bp marker ladder; 2-13, 10^5 , 10^4 , 10^3 , 10^2 , 64, 32, 16, 8, 4, 2, 1, 0 tachyzoites, respectively, diluted in 10^5 rat leucocytes in water. Panel a, primary PCR results; panel b, nested PCR result.

were boiled for 20 min in 0.5-ml conical Treff tubes (Scotlab, Aberdeen), by a method similar to that used by Burg *et al.*⁸ In most instances, PCR was performed at this stage. For DNA extraction, the samples were incubated at 55 °C with shaking for 2 h in lysis buffer (sodium lauryl sulphate 0.5%, 10 mM NaCl, 10 mM disodium-EDTA, 20 mM Tris-HCl, pH 7.5). This procedure was tested both with and without the addition of proteinase K (Sigma), final concentration 200 µg/ml. The product was successively extracted with water-saturated phenol:chloroform (1:1), then chloroform:isoamylalcohol (24:1) and finally precipitated with three volumes of ethanol.

PCR

The PCR was performed in a total sample volume of 50 µl—20 µl of test specimen plus 30 µl of a reagent mix of the following: water, 10× buffer concentrate, deoxynucleoside triphosphates (dNTP), primer S114, primer S115 and TAQ polymerase. The 10× buffer concentrate contained Tris-HCl, KCl, MgCl₂ and gelatine, pH 8.3, and was either produced in-house or supplied by Promega (Southampton). The dNTP contained equimolar dATP, dCTP, dGTP and dTTP (Pharmacia). Primers S114 and S115 were supplied by Severn Biotech, Kidderminster and are equivalent to oligonucleotides 1 and 4 respectively, described by

Burg *et al.*⁸ The TAQ polymerase was purchased from ILS Ltd (London) or Promega. The final concentration of each constituent (after addition of 20 µl of test sample in water) was 10 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, gelatin 0.01%, 0.2 mM each dNTP and 10 µg/ml each primer. Polymerase was added at 0.7 units/sample. Triton X-100 0.1% v/v was included in the final mix, either by addition at 0.25% to the test sample, or supplied in the Promega 10× buffer concentrate. The reaction volumes were overlaid with 100 µl of mineral oil and subjected to 30 temperature cycles of 94 °C for 1.3 min, 53 °C for 2 min and 72 °C for 2.5 min in a Techne PHC-1 Programmable Dri-block (Scotlab) followed by an extension incubation at 72 °C for 5 min.

The 194-bp product was identified by electrophoresis of 16-µl samples (mixed with 4 µl of orange G marker dye 0.01% in glycerol 50% v/v), on agarose (BioRad) 2.5% submarine gels at 133 V for 30–60 min. Twelve or 24 samples were run/gel along with two or four lanes of 123-bp mol. wt marker ladder (Gibco BRL; 3.5 µl including orange G/lane). Bands were stained by ethidium bromide 1 µg/ml added to the electrophoresis buffer (0.089 M Tris-borate, 0.0025 M EDTA, pH 8.3) and viewed and photographed on a UVP transilluminator (Scotlab) with a Polaroid DS34 camera (Genetics Instrumentation, Dunmow) with Type 667 Polaroid film.

Fig. 2. Effect of sample clarification on PCR sensitivity. Electrophoresis results following two-stage primary and nested PCR with primers S114 + S115, then S114 + S331. Lanes 1 and 14, 123-bp marker ladder; 2–7 and 8–13, sample = 10^4 , 10^2 , 32, 8, 2, and 0 tachyzoites in water. Panel a, 2–7, boiled sample homogenates; 8–13, samples clarified at 10600 *g*, 1 min, before nested PCR; panel b, 2–7, samples clarified before primary PCR; 8–13, samples clarified before both PCR stages.

Nested PCR was performed on 0.1 μ l of the product of the primary PCR by testing 20 μ l of a 1 in 200 dilution in water. This optimal dilution was determined in a dilution experiment; under-dilution resulted in competition between the primers in the nested mix and excess from the primary PCR; over-dilution resulted in lower sensitivity. Final buffer concentrations in the 50- μ l PCR sample were the same as in the primary PCR, except that dNTP and polymerase concentrations were halved (0.1 mM each dNTP and 0.35 units/sample, respectively) and different primers (internal S331 + internal P456, or internal S331 + external S114; 5 μ g/ml each) were used. Primers P456 and S331 (Severn Biotech) are equivalent to oligonucleotides 2 and 3, respectively, of Burg *et al.*,⁸ but P456 was 5' labelled with biotin, instead of the T7 RNA polymerase promoter sequence. Nested PCR was subjected to 17 temperature cycles.

Results

Sensitivity

All modifications to the PCR procedure in order to improve its sensitivity were based on dilution experiments of R₁₁ strain tachyzoites in water. Positive

results were regularly attainable with 64–100 parasites after primary PCR and with 1–4 parasites after nested PCR in samples which had been boiled for 20 min in the presence of 10^5 rat, mouse or human leucocytes in water. In fig. 1, a 194-bp band is visible with ethidium bromide stain at all parasite dilutions in rat leucocytes down to 64 parasites after primary PCR, and a 160-bp band to two parasites after nested PCR. Faint bands of other molecular size were frequently visible after primary PCR, but not after nested PCR. In the absence of mammalian leucocytes, similar sensitivity was achieved, but the PCR was inhibited by high parasite numbers ($> 10^4$). Positive results were also possible in the presence of higher leucocyte concentrations; a band was easily visible after nested PCR when six parasites (the lowest number tested) were assayed in the presence of 6×10^5 rat leucocytes, and faintly visible in the presence of 6×10^6 leucocytes.

Small volumes of highly concentrated leucocytes, especially with erythrocyte contamination, produced very viscous and particulate sample homogenates which were difficult to handle. However, clarification by low speed centrifugation markedly reduced test sensitivity (fig. 2). Sensitivity was reduced from between two and eight parasites without clarification to 10^4 parasites after clarification before both PCR steps. The inclusion of Triton X-100 (essential in the reaction

Table I. Time course of R_H infection in Cotton rats and mice

Time after infection (h)	Number of rats	Number of positive results by		Number of mice	Number of positive results by	
		culture*	PCR†		culture*	PCR†
16	3	2	1 (1)	4	2	2 (0)
24	3	1	1 (0)	4	2	4 (2)
40	3	3	3 (3)	4	4	4 (2)
48	3	3	3 (2)	4	3	4 (4)
66	3	2	3 (2)	4	3	4 (2)
Total	15	11	11 (8)	20	14	18 (10)

* Parasites identified in peritoneal exudate of mice 6–10 days after inoculation of specimen.

† Results of water-washed leucocytes compared with those without water wash (in parentheses).

Table II. Time course of infection with Beverley strain in Cotton rats and mice

Time after infection (days)	Number of rats	Number of positive results by		Number of mice	Number of positive results by	
		culture*	PCR†		culture*	PCR†
1	1	0	0 (0)	2	0	0 (0)
2	1	0	0 (0)	2	0	2 (2)
3	1	0	1 (1)	2	0	1 (1)
4	1	0	1 (0)	2	0	2 (2)
7	1	1	1 (1)	2	1	2 (1)
10	1	0	1 (1)	2	0	1 (0)
14	1	1	1 (1)	2	0	1 (1)
17	1	0	1 (1)	2	0	1 (1)
21	1	0	1 (0)	2	1	1 (0)
24	1	1	0 (0)	2	0	1 (0)
28	2	0	2 (1)	2	0	2 (0)
31	1	1	1 (1)	2	0	1 (0)
35	1	0	0 (0)			
38	1	0	1 (1)			
Total	15	4	11 (8)	24	2	15 (8)

*, † See footnotes to table I.

buffer, especially with Promega polymerase) during sample boiling did not release more DNA into the supernate.

When dilutions of R_H tachyzoites in the presence of 10^5 rat leucocytes were extracted with detergent at 55 °C, then organic solvents, the maximum sensitivity after nested PCR was 16 parasites. There were problems in achieving consistent recoveries from small samples with this method, and in some experiments the final sensitivity was considerably poorer than this figure. At the primary PCR stage the maximum sensitivity was 10^3 parasites. Sensitivity was not improved by including proteinase K in the lysis buffer. In the absence of rat leucocytes, parasite DNA was not detectable at any dilution tested from 10^4 downwards.

Specificity

A positive nested PCR result confirmed the specificity of the primary PCR product. Bands of 160 bp with nested primers S114 and S331, or 97 bp with S331 and P456, were seen consistently with positive control samples known to contain toxoplasma R_H tachyzoite DNA. Uninfected leucocyte negative controls pro-

duced no bands. In addition, the primary PCR product was transferred by Southern blotting to a Genescreen plus nylon membrane (Dupont UK, Stevenage) and hybridised with primer S331, labelled with digoxigenin-dUPT by a 3' end-labelling kit (BCL Ltd, Lewes) according to the manufacturer's instructions. In positive control samples, a 194-bp band was detectable by anti-digoxigenin immunoassay performed according to kit instructions.

Adenovirus, cytomegalovirus, herpes simplex virus, *S. aureus*, *C. albicans* and *P. carinii* at all concentrations tested in the presence of 10^5 rat leucocytes produced negative results. In each case, a positive control of 10^2 toxoplasma tachyzoites added to the highest pathogen concentration yielded a visible band.

R_H strain DNA in rat and mouse blood

PCR and isolation in mice were compared for detection of toxoplasma infection in leucocyte fractions from Cotton rat or mouse (BK:TO strain) blood samples, collected at five time intervals after intra-peritoneal infection with R_H strain (table I). The methods had similar sensitivity with both rat and

mouse blood. The results reported for PCR are those which were obtained after washing the leucocytes with water to reduce haemoglobin content. Fewer positive results were obtained if this step was omitted (table I). Both total sample volumes collected and the yield of leucocytes varied considerably throughout the experiment. With rats, positive PCR results were obtained from samples with leucocyte content in the range $(0.4\text{--}3.0) \times 10^5$. With mice, the corresponding figures were $(0.18\text{--}3.0) \times 10^5$ leucocytes. In a repeat R_H strain time course, employing two rats at each time point, the number of positive PCR results increased from 7 of 10 to 8 of 10 when the leucocyte concentration in the sample was adjusted to fall within this range (1×10^5 leucocytes in eight cases; 0.5×10^5 and 0.3×10^5 , respectively, in two cases where the starting concentration was $< 1 \times 10^5$). The original leucocyte content of the sample that became positive was 12.3×10^5 ; in the two that remained negative, it was 9.2×10^5 and 6.0×10^5 , respectively; and the remainder were within the range $(0.3\text{--}2.5) \times 10^5$. In animal culture, 7 of 10 specimens gave positive results.

Beverley strain DNA in rat and mouse blood

Blood samples collected from mice and rats at 12 or 14 time intervals, respectively, after infection produced more positive results in PCR than in animal culture (table II). Again, sample volumes and leucocyte yields were variable. The ranges for positive PCR results were $(0.7\text{--}23.0) \times 10^5$ leucocytes from rats and $(0.3\text{--}4.0) \times 10^5$ from mice.

Discussion

Samples containing cells are normally digested with proteinase K and detergent, then extracted with organic solvents in preparation for PCR assays.^{5, 8, 12} However, DNA extraction with organic solvents involves a risk of losing any pathogen DNA which may be present in small samples.¹³ Our results with dilution series of R_H parasites confirmed this problem, especially when dilutions were carried out in the absence of rat leucocytes. Our ability to detect repeatedly one to four parasites in the presence of rodent leucocytes after simply boiling in water suggests that there is no need to use more elaborate DNA extraction procedures. The concentration of leucocytes added in most dilution experiments was $10^5/20 \mu\text{l}$ but it was also possible to detect six parasites in 6×10^6 leucocytes, which is within the normal range for a 1-ml sample of human blood. As the parasites are likely to be present as infected cells¹ rather than free tachyzoites, with ≥ 16 parasites/cell¹⁴ the test is capable of detecting one infected cell in a 1-ml sample. Enhancing this sensitivity level may be difficult because attempting to test a leucocyte sample concentrated from 10 ml of blood could result in PCR inhibition and it would increase the technical difficulty of

handling small heterogeneous preparations. It was found to be important to test the whole sample; sensitivity was reduced if samples were clarified at any stage.

Toxoplasma DNA was also detected in leucocytes from experimentally infected animals. Combining all these experiments, the range of leucocyte concentrations within which positive PCR results were obtained was $(0.18\text{--}23.0) \times 10^5/\text{sample}$. However, there was some evidence that the parasite DNA may be more easily detectable when the leucocyte content was $< 10^6/\text{sample}$. Therefore, we consider it advisable to test samples with $> 10^6$ leucocytes at more than one dilution. Significant erythrocyte contamination magnified the specimen handling problems and this frequently amounted to > 10 times the leucocyte content. Haemoglobin is claimed to inhibit PCR.¹² A final wash with water was necessary to remove haemoglobin from leucocyte preparations. The problem was demonstrated by the failure to detect R_H strain DNA in some specimens subsequently found to be positive by animal culture, unless this water wash stage was performed. High erythrocyte contamination ($> 10 \times$ leucocyte concentration) was present in 10 (59%) of 17 specimens that produced negative results in the absence of a water wash, compared with only 3 (19%) of 16 that produced positive results. Counts were not available from two positive specimens. Parasitaemia was demonstrated throughout R_H strain infection (16–66 h), which is not surprising in a virulent infection. Even though PCR was no more sensitive than animal culture, parasitaemia was detected earlier and at least as effectively as reported elsewhere.^{6, 12} PCR was an improvement over animal isolation for the samples from rats or mice infected with the Beverley strain. This probably results from decreased sensitivity of the culture method rather than improved PCR sensitivity and reflects the relative virulence of the strains. Small numbers of the virulent R_H strain are more likely to result in demonstrable infection in animals. However, as cyst-forming strains like the Beverley strain are the type usually isolated from human infections, the greater sensitivity of PCR is encouraging.

The detection of toxoplasma in blood at 2–38 days after infection with the Beverley strain indicates that parasitaemia is not transient in animals. It suggests that PCR may still provide valuable diagnostic information in human infections several weeks after onset.

The test as described is fairly simple to perform and could provide results within 10 h of specimen receipt. Its sensitivity and specificity are currently high and there is scope for enhanced sensitivity through biotin-avidin enzymatic amplification methods¹⁵ via the biotin-labelled internal primer after nested PCR. The efficacy of the technique on human blood samples from patients with clinical toxoplasmosis was concurrently investigated.¹⁶ If PCR compares as favourably with animal culture as it does in this study, it

should replace the latter technique as the method of choice for demonstrating the presence of *T. gondii* in clinical specimens, particularly blood.

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