Comparison of two gene amplification methods for the detection of *Toxoplasma gondii* in experimentally infected sheep

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Summary. Efferent lymph and peripheral blood collected from sheep experimentally infected with *Toxoplasma gondii* strain S48 were analysed for parasite DNA by amplification of the B1 and P30 *T. gondii* genes by the polymerase chain reaction (PCR). The relative sensitivity of these two gene amplification methods was assessed and compared with parasite detection by mouse injection (MI). B1 PCR was consistently more sensitive than P30 PCR and the results agreed closely with those from MI. By contrast, P30 PCR gave more than twice as many false negatives results than B1 PCR. The few apparent false positive results given by either PCR method were probably due to the inability of MI to detect non-viable parasites. All specimens collected before infection with *T. gondii* gave negative results by PCR and MI. Parasite DNA was detected by both B1 and P30 PCR in the lymph node of a sheep 12 days after infection but not in other tissues. The results permit a direct comparison between *T. gondii* detection by P30 and B1 PCR. Moreover, they further confirm the value of PCR detection of toxoplasma as a sensitive, specific and reliable diagnostic and research tool.

Introduction

Toxoplasmosis is a zoonosis caused by the protozoan parasite *Toxoplasma gondii* which has social and economic relevance to both human and animal medicine. Although for most healthy individuals infection is mild with few or no clinical symptoms, for vulnerable groups, such as immunocompromised individuals or the developing fetus, toxoplasmosis may prove life-threatening. Therefore, rapid and accurate diagnosis is important. Serologically-based diagnostic techniques for patients who have serious immune dysfunction due to AIDS or through immunosuppressive drug therapy may prove unreliable because of impaired antibody production. Isolation of the parasite by mouse injection (MI) is time consuming and potentially hazardous.

The ability of gene amplification by the polymerase chain reaction (PCR) to detect small amounts of DNA suggests that it may prove to be a sensitive method for the diagnosis of toxoplasmosis. Methods based on amplification of either the P30 gene¹ or the B1 gene² of *T. gondii* have been developed and used for the detection of toxoplasma in various clinical specimens. B1 gene amplification has been used to detect *T. gondii* in a limited number of specimens collected post-mortem from immunocompromised patients and aborted human fetuses,^{3,4} as well as to diagnose

toxoplasma infection *in utero*, suggesting its potential as a method for pre-natal diagnosis of congenital infection.⁵ P30 gene amplification has also been used to detect *T. gondii* in human fetal blood and amniotic fluid^{6,7} and in brain tissue collected by biopsy from AIDS patients.⁸

Turner and Savva⁹ and Turner *et al.*¹⁰ have reported, in uncontrolled field studies, the use of P30 gene amplification to identify equine ocular toxoplasmosis and to detect the parasite in organs and tissues of lambs aborted apparently following maternal toxoplasmosis. To date, however, the use of PCR for the detection of ovine toxoplasmosis has not been assessed under controlled experimental conditions. Furthermore, no attempt has been made to compare directly the relative efficacy of the P30 and B1 PCR detection methods.

A comparison of the B1 and P30 genes as targets for amplification by PCR for the detection of *T. gondii* in experimentally infected sheep is described in this paper. The study aimed to establish (1) whether one of the two PCR methods was more sensitive and to assess the extent of agreement with parasite detection by MI; (2) whether parasites could be detected in ovine peripheral blood as well as in lymph; (3) whether parasites could be detected in ovine solid tissues.

Materials and methods

Parasites

T. gondii strain S48 tachyzoites (kindly supplied by H. Bos, Intervet B.V., The Netherlands) were grown in female Swiss White mice. The parasites were harvested with a 26 gauge needle and syringe from the peritoneal cavities of mice that had been infected 3 days earlier with 10^6 tachyzoites by intraperitoneal injection. Tachyzoites were washed three times by suspension in 0.3 M phosphate-buffered saline (PBS), pH 7.4, followed by centrifugation at 500 *g* (5 min) and counted with a haemocytometer at a magnification of 400. Preparations with < 2% host cell contamination were used for infecting sheep.

Experimental samples

Lymph was obtained from sheep undergoing sampling for concomitant immunological analysis. Efferent lymph draining from the pre-femoral lymph node was collected by cannulation of the duct from six ewes seronegative for *T. gondii*.^{11,12} Each animal was infected with 10^5 *T. gondii* strain S48 tachyzoites¹³ by subcutaneous (s.c.) injection into the flank adjacent to the cannulated node. Lymph was examined before infection and daily thereafter up to 15 days post-infection. Each lymph sample was divided into three and tested for *T. gondii* by MI and by B1 and P30 PCR.

Peripheral blood was collected daily from a further group of three ewes which were initially seronegative for toxoplasma, before and after infection with 10^5 *T. gondii* strain S48 tachyzoites s.c. The lymph nodes of these animals were not cannulated.

A ewe from which the flow of lymph had stopped prematurely after infection with 10^5 *T. gondii* strain S48 tachyzoites s.c., was killed 12 days post-infection and samples (c. 1 g) of brain, spleen, diaphragm, liver, kidney and left and right pre-femoral lymph nodes were removed and prepared for PCR analysis.

PCR

Preparation of samples. Lymph was diluted 1 in 10 with PBS to prevent clotting and centrifuged at 800 *g* for 10 min. Contaminating erythrocytes (RBC) were lysed by the addition of 1 ml of 10 mM Tris-NH₄Cl and the remaining cells were washed three times by resuspension in PBS and centrifugation at 800 *g* for 10 min. The resulting pellet was resuspended in 50 μ l of 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂ containing proteinase K 100 μ g/ml and Tween 20 0.5%. After incubating the mixture at 55°C for 1 h, proteinase was inactivated by boiling and the specimens were stored at -20°C.

Whole blood was treated to remove RBC. Leucocytes were separated from RBC by centrifugation over Lymphoprep (Nycomed, Oslo, Norway) and remaining RBC were lysed with Tris-NH₄Cl as above.

Leucocytes prepared from blood were treated with proteinase K and stored at -20°C.

Tissues were frozen before preparation. Each tissue was finely chopped and washed in PBS. Remaining RBC were lysed and the tissues were digested overnight at 37°C in 100 μ l of 50 mM Tris (pH 8.5), 1 mM EDTA, Tween 20 0.5% containing proteinase K 200 μ g/ml. After digestion, the proteinase was inactivated by boiling and samples were stored at -20°C.

Reaction conditions. PCR was performed in 50- μ l volumes in a Techni PHC3 thermal cycler. Amplification of the P30 gene was performed according to the method of Savva *et al.*¹ with a reaction mixture consisting of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, gelatin 0.01%, 0.2 mM dNTPs (Pharmacia), 0.2 μ M each primer (Oswel DNA services, Edinburgh) and 2.5 units of Taq polymerase (Boehringer Mannheim, Germany). Test sample (5 μ l) was added to the reaction mixture and a 30 cycle amplification process of 1 min at 95°C, 1.5 min at 60°C and 3 min at 74°C was performed with primers DS29 and DS30, followed by a further 15 cycles with nested primers DS38 and DS39 to give a final product of 522 bp. DNA was detected at concentrations of 0.1 pg which gave a sharply defined band corresponding to about 500 bp on an agarose 0.8% gel. Sensitivity was increased by Southern blotting to detect 0.05 pg of DNA, corresponding approximately to the DNA content of one tachyzoite.¹

Amplification of the B1 gene was performed by a method modified from that of Burg *et al.*² The reaction mixture contained 10 mM Tris (pH 8.3), 2.5 mM MgCl₂, 40 mM KCl, gelatin 0.01%, 0.1 mM dNTPs, 0.2 μ M of each primer and 2.5 units of Taq polymerase. Primers P1, P3 and P4 were as described by Burg *et al.*² but P2 consisted of only 20 bases, corresponding to nucleotides 757-776 of the B1 gene (Oswel DNA services). Amplification was performed with P1 and P4 to give a 193-bp product over 25 cycles of 93°C for 1 min, 50°C for 1.5 min and 72°C for 3 min. Amplification products were then diluted 1 in 20 in distilled water to reduce amplification of non-specific products and amplified again with nested primers P2 and P3 over 15 cycles to give a 94-bp product. With this method, 0.05 pg of DNA could be seen on an agarose 1% gel although resolution was much improved on a polyacrylamide 7.5% gel.

Negative controls consisting of distilled water and uninfected lymph and blood were run for each set of tests to monitor cross-contamination. General precautions to prevent cross-contamination included the use of positive displacement pipettes, disposable plastic ware and the wearing of latex gloves.

DNA extraction for controls. For positive controls, 10^9 *T. gondii* strain S48 tachyzoites were lysed in 50 mM Tris (pH 8.0), 50 mM EDTA, SDS 1%, containing proteinase K 100 μ g/ml. After incubation for 3 h, the nucleic acid was extracted with phenol-chloroform, then precipitated with sodium acetate-ethanol at -20°C and pelleted by centrifugation at

10000 *g* for 15 min. The DNA pellet was washed in ethanol 70% and resuspended in TE (10 mM Tris, 0.1 mM EDTA, pH 7.6). RNA was removed by incubation with RNAase 20 µg/ml for 30 min at 37°C and the DNA was re-extracted and precipitated as above. The pellet was resuspended in 100 µl of TE and the DNA quantified by measuring UV absorption at 260 nm.

For negative DNA controls, DNA was extracted by the above method from *Sarcocystis* sp. (kindly supplied by N. Lally, Centre for Tropical Veterinary Medicine, Edinburgh) and from *Pneumocystis carinii* obtained from the lungs of rats.¹⁴ DNA extractions from *Candida albicans* and *Aspergillus niger* were also performed.^{15,16} No amplification products were obtained from the DNA of any of these organisms with either P30 or B1 PCR.

Electrophoresis and Southern blotting

Amplification products were analysed routinely on agarose gels. Southern blotting was used to confirm the identity of the B1 and P30 PCR products and to distinguish these from non-specific products occasionally apparent when B1 and P30 PCR was performed on lymph samples. For Southern blotting, DNA products were transferred to Hybond[®] membranes (Amersham, Bucks) and DNA-DNA hybridisation was performed with digoxigenin-labelled fragments produced from DS 38 and DS 39 for P30 products and from P2 and P3 for B1 products (DNA labelling and detection kit, Boehringer Mannheim).

Detection of T. gondii in lymph by mouse injection

Samples (250 µl) of each specimen of lymph tested were injected intraperitoneally into each of three mature female Swiss White mice. Animals were inspected daily and those which appeared ill were killed; a sample of peritoneal exudate was removed and inspected microscopically for tachyzoites. Serum was collected from mice which survived at 8 weeks after injection and was tested for the presence of toxoplasma antibodies by IgG ELISA.¹⁷

Antibody detection

Blood was collected daily from sheep and the serum was examined for *T. gondii*-specific antibody by ELISA.¹⁷

Statistical analysis

Data for parasite detection in lymph by PCR and MI was analysed by a generalised linear model¹⁸ with a binomial error term. This was programmed in the Genstat 5 programming language.¹⁹ Parasite detection in blood by B1 and P30 PCR was compared by a two-tailed *t* test.

Results

Parasite detection in efferent lymph

A total of 101 lymph specimens was collected from 4 days before infection up to 15 days post-infection and analysed for *T. gondii* by each detection method. The numbers of positive tests given by MI, and B1 and P30 PCR are shown in table I. *T. gondii* was isolated by MI from 29 samples collected between 3 and 12 days post-infection compared with 30 that were positive by B1 PCR and 20 by P30 PCR. For individual sheep, the mean total number of days in which samples gave positive results was 4.8 (SD 3.2) days with MI, 5.0 (SD 1.9) days with B1 PCR and 3.2 (SD 2.2) days with P30 PCR. *T. gondii*-specific antibody was not detected in the serum of any mouse that survived after injection of lymph.

The performance of B1 and P30 PCR detection was compared with MI for each lymph sample. Twenty-three tests that were positive by MI were also positive by B1 PCR, whereas P30 PCR gave 14 positive tests that were confirmed to be positive by MI. The numbers of false negative results, i.e., samples that were shown to be positive by MI but gave negative PCR results, were 15 with P30 PCR only but six with B1 PCR. Both PCR methods gave a small number of apparent false positive results (seven and six for B1 and P30 respectively); most of these occurred towards the end of infection. An estimate of the overall reliability of both B1 and P30 PCR detection compared with MI for all 101 samples showed an 87% agreement between MI and B1 PCR compared with 79% with P30 PCR. When the frequency of parasite detection by B1 or P30 PCR was compared with only those 29 samples shown to be positive by MI, the agreement between PCR and MI was 79% for B1 PCR but only 52% for P30 PCR.

The results for the B1 and P30 methods were compared further by calculating the number of positive results scored for each method out of a total delimited by the "window" of positive results given by MI for each animal. After removing between-sheep variation, the difference between the B1 and P30 methods was statistically significant ($F_{1,4} = 9.320$; $p = 0.038$).

Parasite detection in blood

Both the B1 and P30 PCR methods detected *T. gondii* DNA in the peripheral blood of the three sheep during infection with tachyzoites of strain S48. B1 PCR detected parasites over a mean of 4.7 (SD 0.6) days compared with 1.7 (SD 0.6) days with P30 PCR (table II). In each case, parasitaemia was detected earlier with B1 PCR and over a significantly longer time ($p = 0.003$, 4 df, two-tailed *t* test). The infection became established in each of these animals and seroconversion took place after 11 days. Having established the close agreement between MI and B1 PCR in the previous experiment, isolation of the

Table I. Comparison of B1 PCR, P30 PCR and MI for detection of *T. gondii* in lymph from infected sheep

Days post-infection	Number of sheep tested	Number of positive tests by		
		MI	B1 PCR	P30 PCR
-4	2	0	0	0
-3	4	0	0	0
-2	5	0	0	0
-1	5	0	0	0
0	6	0	0	0
1	6	0	0	0
2	6	0	0	0
3	6	2	1	2
4	6	4	4	5
5	6	4	6	3
6	6	4	5	2
7	6	5	4	2
8	5	4	4	2
9	5	4	3	2
10	5	1	1	1
11	5	1	1	1
12	5	0	1	0
13	5	0	0	0
14	5	0	0	0
15	2	0	0	0
Total	101 (tests)	29	30	20

Table II. Detection of *T. gondii* in the blood of three sheep by PCR and antibody detection by ELISA after experimental infection with 10^5 tachyzoites of strain S48

Sheep	Test	Result on days post-infection													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	B1 PCR	-	-	-	-	-	+	+	+	+	+	-	-	-	-
	P30 PCR	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-	-	+	+	+	+	+
B	B1 PCR	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	P30 PCR	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-	-	+	+	+	+	+
C	B1 PCR	-	-	+	-	-	+	+	+	-	-	+	-	-	-
	P30 PCR	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	ELISA	-	-	-	-	-	-	-	-	-	+	+	+	+	+

parasite by MI was not attempted for blood or tissue samples.

Parasite detection in lymph node tissue

The left and right pre-femoral lymph nodes from the sheep killed 12 days after infection were shown to be positive for *T. gondii* by both B1 and P30 PCR. Parasite DNA was not detected in the liver, spleen, brain, kidney or diaphragm by either method.

Discussion

In this study, amplification of either the B1 or P30 gene by PCR detected *T. gondii* in lymph, blood and lymph node samples from experimentally infected sheep, but the relative sensitivity of the two methods differed. In lymph, the agreement between parasite detection by MI and PCR was significantly higher with

B1 PCR than with P30 PCR. Detection by P30 PCR gave almost twice as many false negative results as B1 PCR. Furthermore, parasites were detected in the peripheral blood of infected sheep both earlier and for a significantly longer period with B1 PCR than with P30 PCR.

Both B1 and P30 gene amplification gave a similar, but low, number of apparent false positive results. However, these may represent samples containing parasite DNA but no viable organisms since, unlike MI, PCR does not rely on live parasites to give a positive result. As none of the mice which survived until 8 weeks after injection with lymph produced antibody against *T. gondii*, it is concluded that only those that developed clinically apparent disease were exposed to tachyzoites. Apparent false positive results which occurred towards the end of infection may result from samples containing traces of parasite DNA but no viable tachyzoites. Furthermore, no positive

PCR results were obtained in any of the lymph samples collected before infection or in any other negative control. The discrepancies between negative MI tests and positive PCR results were, therefore, probably due to constraints of MI detection rather than to genuine false positive PCR tests.

Although detection by amplification of the B1 gene was repeatedly more sensitive than that of the P30 gene, the absolute sensitivity of each method when applied to ovine lymph and blood remains to be determined. Moreover, a comparative investigation into the efficacy of P30 and B1 PCR with other clinical material, including human specimens, is required before any conclusions can be made about the use of either target gene for parasite detection in general. The preparations of whole blood for PCR by removal of RBC with Lymphoprep may require further improvement as this method would not isolate free parasites or those within cells remaining in the RBC fraction after centrifugation. Although this method minimises contamination of the specimen with haemoglobin it is time consuming and requires immediate treatment of samples before they can be frozen and stored.

The sensitivity of B1 amplification may be due to the repetitive nature of the B1 gene, of which 25–50 copies are present in the genome of *T. gondii*.²⁰ Brezin *et al.*²¹ suggested that this might account for their successful use of B1 amplification to detect human ocular toxoplasmosis but failure with P30 PCR. Since amplification of a single specific fragment is sufficient to give a positive result, the repeated short DNA fragments of the B1 gene provide a better target than the longer single copy P30 gene. However, where the danger of cross-contamination is greater, the superior sensitivity of B1 PCR will require greater stringency in the laboratory to avoid predisposing the diagnosis to false positive results.

Both P30 and B1 PCR detected *T. gondii* in the lymph nodes from an infected animal even though the tissue had been frozen and thawed before analysis.

This contrasts with an earlier report by Weiss *et al.*²² who found that parasite detection in lymph node tissue frozen before analysis was unreliable compared with detection in paraffin-embedded sections. The reason for this discrepancy is not known. In the present study, the parasite was not detected by PCR in tissues other than lymph node. While this may reflect the genuine distribution of tachyzoites throughout the host, the possibility that freezing and thawing of some tissues may compromise the sensitivity of PCR cannot be excluded without further investigation of a larger number and range of tissue specimens.

By demonstrating the efficacy of PCR detection of *T. gondii* in a large number of clinical specimens under controlled experimental conditions, this study confirms the potential of PCR as a valuable technique for the detection of the parasite and suggests the value of an ovine model for the assessment of PCR diagnosis of human toxoplasmosis. For instance, the ability to monitor tachyzoites in peripheral blood may be of use in the detection of reactivated human toxoplasmosis associated with immune dysfunction. Moreover, the capability of PCR to monitor the kinetics of *T. gondii* in body fluids such as lymph and blood suggests that PCR will prove a valuable experimental tool for the study of the pathogenesis of toxoplasmosis in both man and animals. In these instances, B1 PCR would seem to be the method of choice because of its exquisite sensitivity that is not apparently compromised by false positive results. Furthermore, the sensitivity of PCR will enable gene amplification to replace MI as the standard method for direct detection of *T. gondii*, thus dispensing with the use of large numbers of mice.

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