

Technical report

Quantitation of *Toxoplasma gondii* DNA in a competitive nested polymerase chain reaction

P Y C Lee, J Mangan, R E Holliman, P D Butcher

Abstract

Aim—To quantify *Toxoplasma gondii* DNA using a specially constructed artificial template as competitor in a nested polymerase chain reaction (PCR).

Methods—The diagnostic assay was a nested PCR employing four primers that amplify part of the single copy gene for the P30 major surface antigen in *T gondii*. An artificial competitor containing the four primer binding sites was made first by creating a 216 bp deletion in the native 914 bp full length PCR product using restriction enzyme digestion, ligation of selected digestion fragments, and cloning the ligation product into an *E coli* plasmid vector for production. Competitive nested PCR using three different quantities of *T gondii* genomic DNA with four corresponding 10-fold dilutions of the artificial competitor was then performed, and the results visualised with agarose gel electrophoresis. A standard curve was drawn by plotting the *T gondii* to competitor ratio readings against \log_{10} of the competitor readings.

Results—The band intensities on agarose gel showed quantitative amplification in competitive nested PCR. The amount of competitor required to achieve equal molar amounts of PCR products is calculated by reading off the value of the competitor where the *T gondii* to competitor ratio equals 1 on the standard curves.

Conclusions—Competitive PCR is possible with a nested assay, and quantitative amplification is well preserved. The use of an artificial competitor containing the same primer binding sites as the target enables the absolute amount of *T gondii* DNA in unknown samples to be estimated. In addition, the competitor simultaneously serves as a control for detecting false negative results of failed reactions in individual assay runs.

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Keywords: *Toxoplasma gondii*; DNA quantitation; competitive nested polymerase chain reaction

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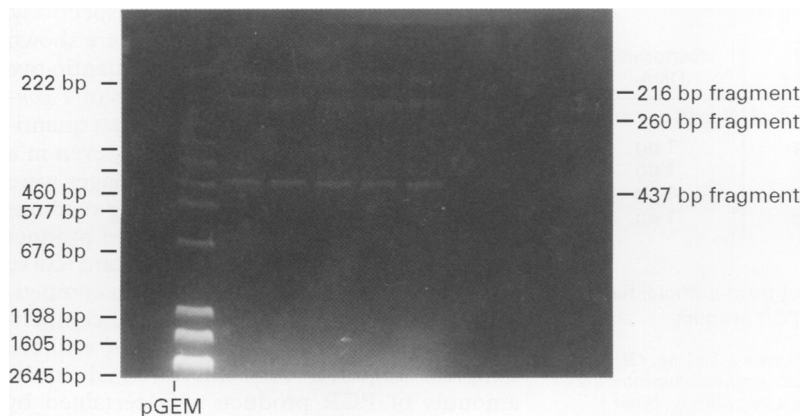


Figure 1 Digestion products of native 914 bp polymerase chain reaction product. Restriction enzyme digestion with *BsiHKA1* at 60°C results in fragment sizes of 216, 260, and 437 bp.

The protozoan parasite *Toxoplasma gondii* is a well recognised cause of severe and life threatening infection in immune compromised individuals such as the fetus, AIDS patients, and organ transplant recipients. In these groups, serological investigation may be unreliable and parasite isolation techniques may not achieve the necessary level of sensitivity or rapidity.¹ Amplification of a number of target genes by the polymerase chain reaction (PCR) has been used for the rapid and highly sensitive detection of the parasite²⁻⁴ and the technique applied to the diagnosis of human toxoplasma infection.⁵ There is, however, no agreement as to the preferred PCR target for the diagnosis of toxoplasmosis.

Currently available PCR tests do not distinguish between active infection and the presence of quiescent cysts, nor do they provide information on the viability of the parasite.⁶ This deficiency could be addressed by quantitative PCR. Some clinical samples remain inhibitory to the polymerase enzyme after DNA extraction and generate false negative PCR findings. This phenomenon can be detected by the addition of a positive control to each test reaction.⁷ We developed an artificial template as a competitor in a nested PCR which allows the quantitation of target *T gondii* DNA in the clinical sample and the detection of PCR inhibition in the reaction mix. This approach can be applied to the range of PCR assays in current clinical use.

Methods

MATERIALS

The diagnostic PCR assay in use amplifies part of the P30 gene of the parasite's DNA which codes for the major surface antigen P30 (a sin-

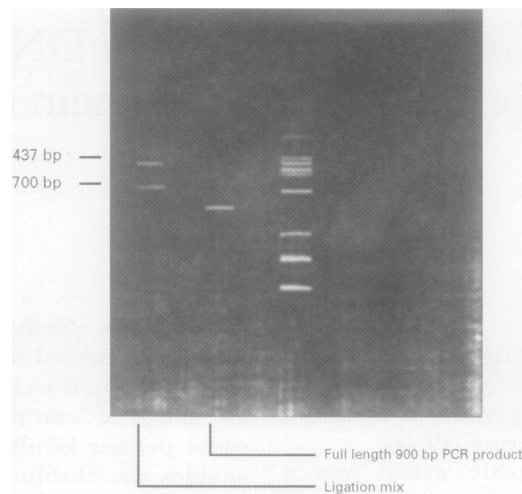


Figure 2 Ligation of *BsiHKA1* digested fragments. Fragment sizes 437 and 260 bp were ligated overnight with T_4 DNA ligase and ligation mix electrophoresed to confirm successful ligation by the presence of a 697 bp fragment. PCR, polymerase chain reaction.

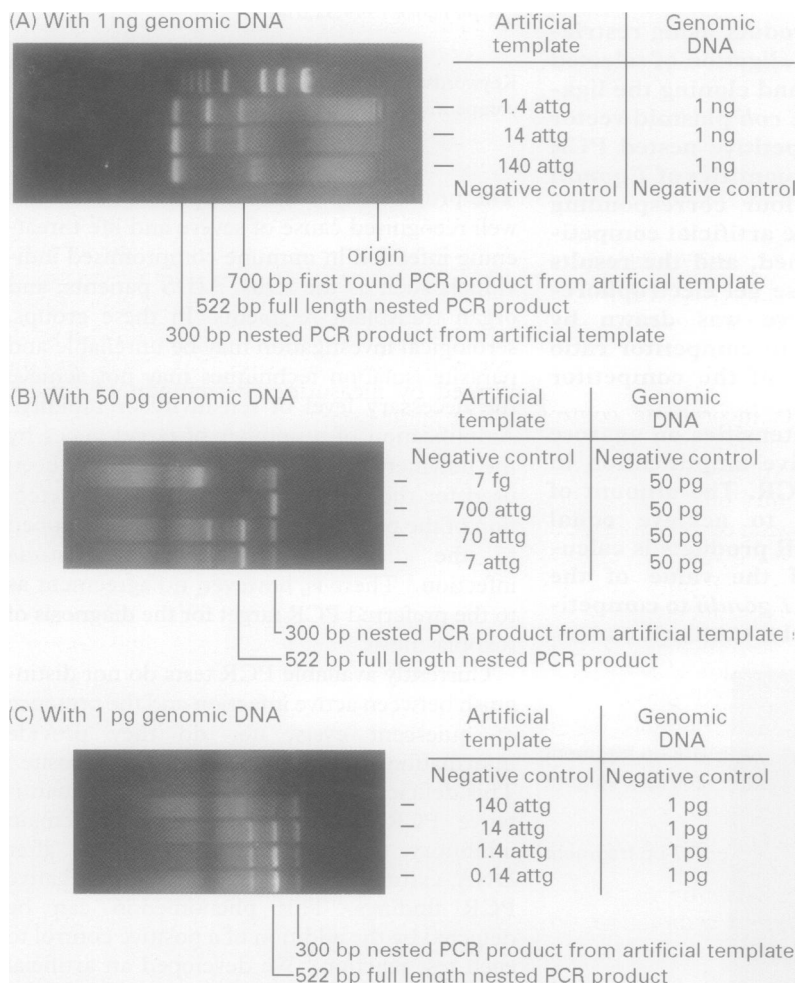


Figure 3 Competitive nested polymerase chain reaction (PCR) with (A) 1 ng, (B) 50 pg, and (C) 1 pg of genomic DNA. PCR products from the artificial template/competitor are 697 bp from the first round and 306 bp from nested amplification, respectively. Band intensities for the 306 bp product (resultant from amplification of the artificial template) are inversely proportional to those for the 522 bp product (resultant from amplification of genomic DNA) and directly proportional to the increasing amounts of artificial template, demonstrating good preservation of the quantitative nature of PCR.

gle copy gene). It is a nested PCR assay employing four primers, resulting in 914 bp and 522 bp native amplification products from the first and second round reactions, respectively.⁵ An artificial template was constructed by producing a small deletion of 216 bp in the central portion of the native 914 bp product. This was carried out by restriction enzyme digestion of the 914 bp product with *BsiHKA1* enzyme at 60°C, resulting in digestion product sizes of 216, 260, and 437 bp. Separation of fragments by electrophoresis on low melting point agarose gel was then performed. The two fragments (437 and 260 bp) flanking the central portion (216 bp) were gel purified, ethanol precipitated, and then gel ligated overnight with T_4 DNA ligase in 20 μ l volumes. The ligation products were cloned into *Escherichia coli* containing pCR vector and the colonies containing the 697 bp insert/artificial template within the plasmid were selected for DNA extraction.

COMPETITIVE NESTED PCR

Three fixed quantities of genomic *T gondii* DNA (1 ng, 50 pg, and 1 pg) and four corresponding 10-fold dilutions of the artificial competitor were subjected to PCR in 50 μ l volumes (details of PCR conditions have been published previously⁵). The corresponding quantities of artificial competitor were determined by calculating the amount of DNA that is 70 000-fold less than the parasite's genome, which is approximately 7×10^7 bp as the P30 gene is only approximately 1000 bp. The nested PCR products from this competitive amplification were visualised by ethidium bromide staining of 1% agarose gel electrophoresis, using TAE (40 mM Tris/HCl, pH 7.5, 20 mM sodium acetate, 1 mM EDTA, pH 8.0).

Results

The results of *BsiHKA1* digestion and ligation of the 437 and 260 bp fragments with T_4 DNA ligase are shown in figs 1 and 2. Amplification products from genomic DNA were 914 and 522 bp from the first and second round (nested) PCR, respectively. Amplification products from the artificial template/competitor are 697 and 306 bp from the first and second round (nested) PCR, respectively. The band intensities on agarose gel are shown in fig 3 and show competitive quantitative amplification with all three quantities of *T gondii* genomic DNA. They also show that quantitative amplification is well preserved, even in a nested assay. These polaroid film images were scanned on a desk jet scanner giving readings that quantify the amount of amplified product represented by each band. A standard curve was drawn by plotting the *T gondii* to competitor ratio readings against \log_{10} of the competitor readings (fig 4). The amount of artificial template required to achieve equal molar amounts of PCR products is ascertained by reading off the anti-log value of the competitor where the *T gondii* to competitor ratio equals 1 on the standard curves.

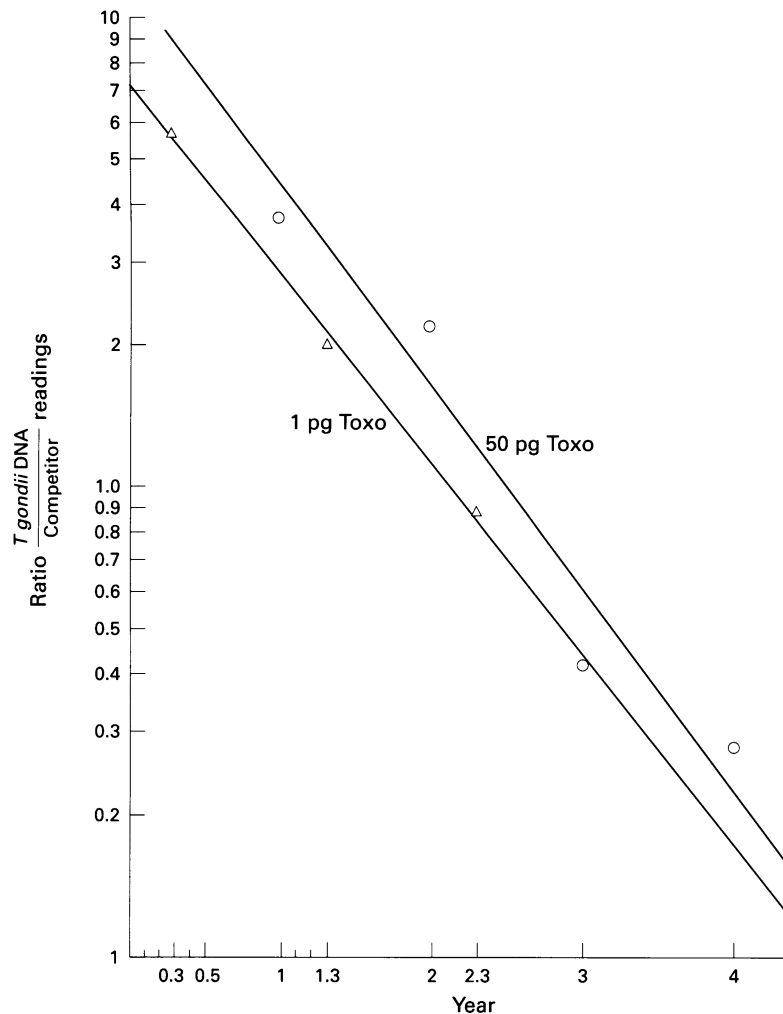


Figure 4 Standard curves. The log of the ratio of amplified *T gondii* DNA to competitor products is graphed as a function of the log of the known amount of competitor added to the PCR reactions. The amount of artificial template/competitor required to achieve equal molar amounts of PCR products and equal band intensities on electrophoresis can be ascertained by reading off the anti-log value of the competitor where the *T gondii* to competitor ratio equals 1 on the x axis.

Discussion

Conventional PCR tests incorporate control positive and negative samples in each assay run.⁵ While this approach will detect gross contamination or global failure of the assay run, individual test failures owing to the presence of inhibitors in a sample are missed. In clinical practice, samples such as CSF and urine are rarely inhibitory but others, including blood and pleural fluids, can be problematic. This issue is addressed by the addition of an artificial template to every well of the PCR assay.

The rapid method for detecting false negative PCR results owing to inhibition or reaction failure has been described for assays to detect other organisms.⁸ A quantitative competitive PCR assay for toxoplasma infection in animals has been described⁹ but the addition of an artificial template to detect inhibitors in clinical samples was not considered. Furthermore, this work used an assay based on a single round of PCR. Many clinical assays for detecting *T gondii* are based on nested primers using two rounds of PCR. We have described the development of this method of using an artificial control template in each reaction for a nested PCR assay which detects the P30 surface protein of *T gondii* tachyzoite. Although

the control template contains an internal 216 bp deletion, which allows it to be distinguished from the natural PCR products by electrophoretic mobility, it is nonetheless amplified by the same primer pairs as the natural template. The results show that it is possible to perform competitive PCR with a nested assay. In addition, the band intensities on agarose gel for the nested reactions show clearly the preservation of the quantitative nature of competitive PCR. This feature can be used to quantify unknown amounts of genomic DNA within clinical samples by first plotting a standard curve with varying known amounts of artificial template against the ratio of genomic DNA to artificial template; then, by incorporating two different quantities of template into clinical samples in duplicate, intensities of the resultant agarose gel bands can be measured and the corresponding quantity of genomic DNA in the clinical sample calculated from the standard curve. This in turn allows the calculation of the numbers of parasites present in a specimen.

We have shown that the addition of predetermined amounts of the artificial template allows estimation of the quantity of target DNA in a specimen. Several potential clinical applications of this approach can be considered. *Toxoplasma* PCR assays in current clinical use produce only a qualitative result. Consequently any positive findings must be interpreted with caution as these may represent the detection of non-viable organisms or quiescent cysts of the parasite.⁶ Positive PCR findings for amniotic fluid taken from mothers whose babies were later shown to be free of infection have been reported.¹⁰ One explanation for this phenomenon would be the presence of non-viable parasites in the amniotic fluid. In contrast, during active infection associated with congenital transmission, the numbers of trophozoites in the amniotic fluid may be increased and this distinction might be confirmed by measuring the amount of parasite DNA in the sample.

Reactivation of toxoplasma cysts is thought to be the pathogenic mechanism of disease associated with AIDS, ocular toxoplasmosis, and organ transplantation.¹ Clinical samples from such patients may contain quiescent cysts in the absence of toxoplasma associated illness. Quantitative PCR may allow the identification of quiescent infection or reactivated disease, a distinction not always possible using conventional, qualitative PCR assays. A preliminary report has suggested quantitative PCR may be useful in the diagnosis of ocular toxoplasmosis.¹¹

Finally, quantitation of parasite DNA in clinical samples may be of prognostic value. Establishing the amount of toxoplasma DNA per unit of, for example, fetal or neonatal blood may give some idea of the likely severity of subsequent disease and handicap.

This study has shown that quantitative measurement of toxoplasma DNA can be performed in the experimental situation. Prospective trials are now required to establish if these results can be reproduced in routine practice and to determine the clinical value of the approach.

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