

Real-Time PCR for Quantitative Detection of *Toxoplasma gondii*

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The protozoan *Toxoplasma gondii* is one of the most common infectious pathogenic parasites and can cause severe medical complications in infants and immunocompromised individuals. We report here the development of a real-time PCR-based assay for the detection of *T. gondii*. Oligonucleotide primers and a fluorescence-labeled TaqMan probe were designed to amplify the *T. gondii* B1 gene. After 40 PCR cycles, the cycle threshold values (C_T) indicative of the quantity of the target gene were determined. Typically, a C_T of 25.09 was obtained with DNA from 500 tachyzoites of the *T. gondii* RH strain. The intra-assay coefficients of variation (CV) were 0.4, 0.16, 0.24, and 0.79% for the four sets of quadruplicate assays, with a mean interassay CV of 0.4%. These values indicate the reproducibility of this assay. Upon optimization of assay conditions, we were able to obtain a standard curve with a linear range (correlation coefficient = 0.9988) across at least 6 logs of DNA concentration. Hence, we were able to quantitatively detect as little as 0.05 *T. gondii* tachyzoite in an assay. When tested with 30 paraffin-embedded fetal tissue sections, 10 sections (33%) showed a C_T of <40 and were scored as positive for this test. These results were consistent with those obtained through our nested-PCR control experiments. We have developed a rapid, sensitive, and quantitative real-time PCR for detection of *T. gondii*. The advantages of this technique for the diagnosis of toxoplasmosis in a clinical laboratory are discussed.

The protozoan parasite *Toxoplasma gondii* has emerged as an important opportunistic infectious pathogen affecting organ transplant recipients, AIDS patients, and other immunocompromised patients. Toxoplasmic encephalitis and extracerebral toxoplasmosis are among the major life-threatening *T. gondii* infections of these patients (4, 6, 19, 29). In addition, toxoplasmic infection during pregnancy may lead to severe, if not fatal, infection of the fetus (7, 11, 25). If the fetus is infected in the first trimester, the result is spontaneous abortion, stillbirth, or severe disease. If infection occurs after the first trimester, disease manifestations include epilepsy, encephalitis, retardation, blindness, and other neurological disorders. Emphasis is placed on preventive measures and early diagnosis of the infection in order to prevent these severe complications of toxoplasmosis.

Current diagnosis of toxoplasmosis relies either on serological detection of specific anti-*Toxoplasma* immunoglobulin, on culture of amniotic fluid or fetal blood, or on other nonspecific indicators of infection (14, 25). Although serological testing has been one of the major diagnostic techniques for toxoplasmosis, it has many limitations. For example, it may fail to detect specific anti-*Toxoplasma* immunoglobulin G (IgG) or IgM during the active phase of *T. gondii* infection, because these antibodies may not be produced until after several weeks of parasitemia. Therefore, the high risk of congenital toxoplasmosis of a fetus may be undetected because the pregnant mother might test negative during the active phase of *T. gondii* infection. Furthermore, the test may fail to detect *T. gondii* infection in certain immunocompromised patients due to the

fact that the titers of specific anti-*Toxoplasma* IgG or IgM may fail to rise in this type of patient (23). An alternative method of identifying *T. gondii* by mouse inoculation or tissue culture of the clinical specimen may confirm the infection by parasites. However, this method usually requires several days to obtain results and is labor-intensive (20). Thus, a more efficient method is needed to provide rapid and quantitative results for the diagnosis of *T. gondii* infection.

Several PCR-based techniques (16, 18, 24) have been developed for the diagnosis of toxoplasmosis using various clinical specimens, including amniotic fluid (3, 11), blood (1, 13, 17), cerebrospinal fluid (27), and tissue biopsy (15). Among these techniques, nested PCR followed by hybridization of PCR products has been the most sensitive method. However, the major disadvantage of these methods is that they are quite time-consuming and do not provide quantitative data. The recent advent of a real-time quantitative PCR technique has proven useful in various applications, including pathogen detection, gene expression and regulation, and allelic discrimination (5, 9, 28). Real-time PCR utilizes the 5' nuclease activity of *Taq* DNA polymerase (12) to cleave a nonextendible, fluorescence-labeled hybridization probe during the extension phase of PCR. The fluorescence of the intact probe is quenched by a second fluorescent dye, usually 6-carboxy-tetramethyl-rhodamine (TAMRA). The nuclease cleavage of the hybridization probe during the PCR releases the effect of quenching resulting in an increase of fluorescence proportional to the amount of PCR product, and can be monitored by a sequence detector, such as the GenAmp 5700 Sequence Detection System (PE Applied Biosystem, Foster City, Calif.). In this study, we describe the development of a real-time quantitative PCR for the detection of *T. gondii*. The use of this methodology will facilitate the diagnosis of *T. gondii* in clinical laboratories.

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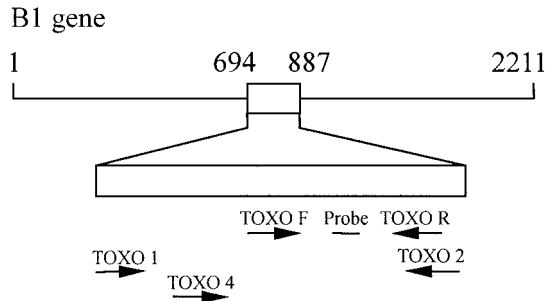


FIG. 1. Design of real-time PCR for detection of the *T. gondii* B1 gene. The relative positions of the primers and TaqMan probe in the B1 gene for real-time PCR and nested PCR are shown.

MATERIALS AND METHODS

Materials. A GenomicPrep cell and tissue DNA isolation kit was purchased from Amersham Pharmacia (Uppsala, Sweden). The TaqMan universal PCR master mix reagent kit, primers, and probe for real-time and nested PCR were purchased from PE Applied Biosystem. The *T. gondii* RH strain tachyzoites were kindly provided by Gan-Nan Chang, Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan, Republic of China. All the paraffin-embedded fetal tissue sections were from the Department of Pathology, Chang Gung Memorial Hospital, Tao-Yuan, Taiwan, Republic of China.

Preparation of DNA templates for PCR. The *T. gondii* tachyzoites were obtained after peritoneal lavage of mice inoculated with the RH strain. Parasites collected from the mouse ascitic fluid were washed and resuspended in phosphate-buffered saline. The concentration of tachyzoites was determined by phase-contrast microscopy using the counting chamber. For preparation of positive control DNA, indicated amounts of *T. gondii* tachyzoites (RH strain) were incubated at 95°C for 10 min to denature the parasite and to release the DNA. The suspension was then used as a positive control in both the nested PCR and the real-time PCR.

High-molecular-weight DNA was extracted from paraffin-embedded tissue sections using a GenomicPrep cell and tissue DNA isolation kit as described by the manufacturer. Briefly, tissue sections were suspended in a cell lysis solution with proteinase K (20 µg/µl). After overnight incubation at 55°C, the lysates were heated at 95°C for 10 min to inactivate proteinase K and then were deproteinated with a protein precipitation solution. The precipitates were removed by centrifugation, and the DNA-containing supernatant was pipetted into a new 1.5-ml

centrifuge tube. The DNA was then precipitated with isopropanol and resuspended in ultrapure water.

Detection of *T. gondii* B1 gene by real-time quantitative PCR. The forward primer (TOXO-F), reverse primer (TOXO-R), and TaqMan probe for real-time PCR amplification were designed with the PrimerExpress software (PE Applied Biosystem) to specifically amplify the *T. gondii* B1 gene. The target DNA for real-time PCR amplification was the published sequence of the 35-fold repetitive B1 gene of the *T. gondii* RH strain (2). Briefly, template DNA was added to a reaction mixture containing 25 µl of 2× PCR universal master mix, 5 µl of the forward primer TOXO-F (5 µM, 5'-TCCCTCTGCTGGCGAAAAGT-3'), 5 µl of the reverse primer TOXO-R (5 µM, 5'-AGCGTTCGTGGTCAACTATC GATTG-3'), and 5 µl of TaqMan probe (2 µM, 6FAM-TCTGTGCAACTTTG GTGATTTCGAG-TAMRA) in a final volume of 50 µl. The PCRs were performed with the GenAmp 5700 Sequence Detection System (PE Applied Biosystem). After initial activation of AmpliTaq Gold DNA polymerase at 95°C for 10 min, 40 PCR cycles of 95°C for 15 s and 60°C for 1 min were performed. The cycle threshold value (C_T), indicative of the quantity of target gene at which the fluorescence exceeds a preset threshold, was determined. This threshold was defined as 20 times the standard deviation of the baseline fluorescent signal, i.e., the normalized fluorescent signal of the first few PCR cycles. After reaching the threshold, the sample was considered positive.

Nested PCR for detection of *T. gondii* B1 gene. Template DNA was added to a final volume of 50 µl of PCR mixture consisting of 5 µl of 10× PCR buffer (50 mM Tris-HCl [pH 9.1], 16 mM ammonium sulfate, 3.5 mM MgCl₂, and 150 µg/ml of bovine serum albumin), 8 µl of 1.25 mM deoxynucleoside triphosphate, 0.5 µl of *Taq* DNA polymerase (5 U/µl), 1.5 µl of 20-pmol forward primer (TOXO 1; 5'-GGAACTGCATCCGTTTCATGAG-3'), and 1.5 µl of 20-pmol reverse primer (TOXO 2; 5'-TCTTTAAAGCGTTCGTGGTC-3'). The mixture was denatured at 94°C for 10 min, followed by 30 PCR cycles of 94°C for 1 min, 60°C for 15 s, and 72°C for 45 s. One microliter of the resulting PCR product was reamplified under identical conditions in a reaction mixture identical in composition to that of the first-round PCR, except that the primer TOXO 1 was replaced with the primer TOXO 4 (5'-TGCATAGGTTGCAGTCACTG-3'). The second PCR product was analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide.

RESULTS

The primers used are shown in Fig. 1. DNA extracted from the *T. gondii* RH strain equivalent to 500 tachyzoites was used as a template for the establishment of this PCR technique. A typical amplification plot (change in fluorescent signal versus cycle numbers) with a C_T of 25.09 was obtained (Fig. 2A). Electrophoretic analysis of the real-time PCR product on a 2% agarose gel showed an expected 98-bp band (Fig. 2B). DNA

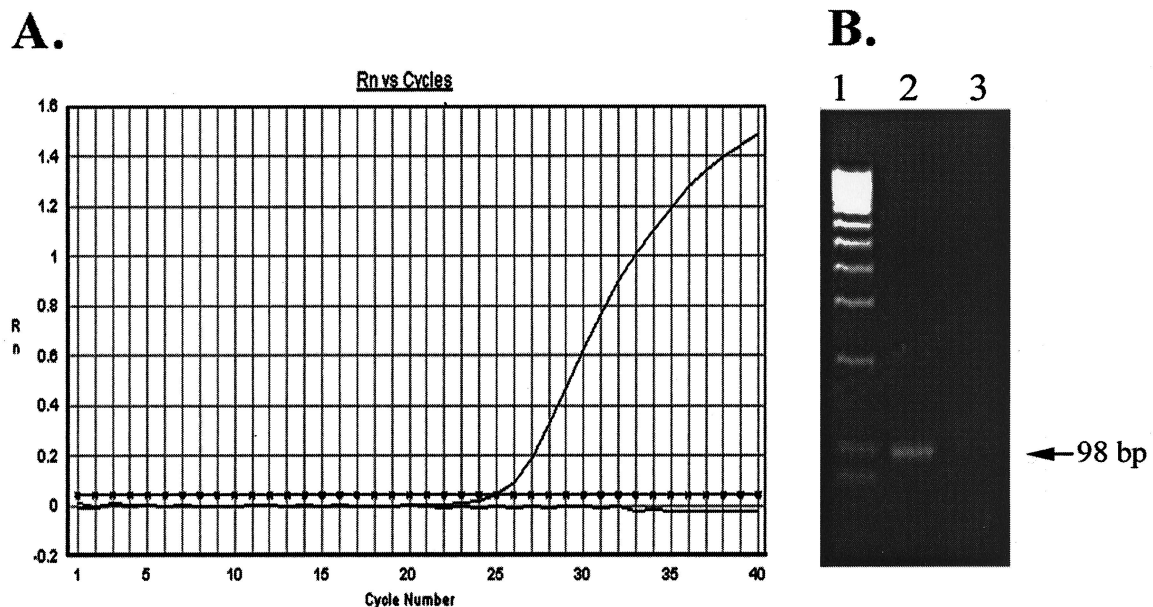
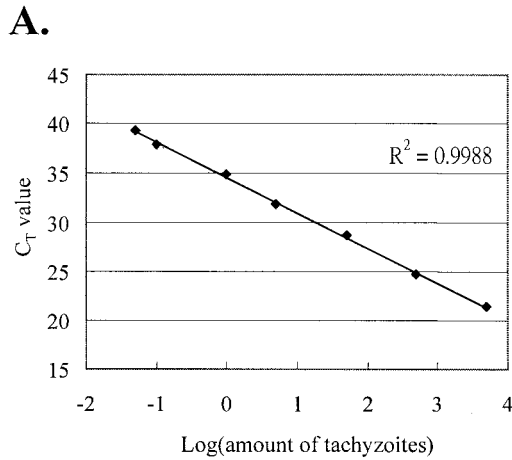


FIG. 2. Real-time PCR detection of the *T. gondii* B1 gene. (A) Typical amplification plot with 500 tachyzoites as the initial DNA template. Rn, fluorescent signal. (B) The PCR product from panel A was fractionated on a 2% agarose gel followed by visualization with ethidium bromide staining. Lane 1, DNA molecular weight marker; lane 2, 500 tachyzoites; lane 3, no-template control.



B.

Tachyzoites	C _T
5000	21.38
500	24.71
50	28.69
5	31.88
1	34.86
0.1	37.80
0.05	39.34
NTC	40.00

FIG. 3. Establishment of the standard curve for quantification of *T. gondii*. Serial dilutions of *T. gondii* DNA, ranging from 5,000 to 0.05 tachyzoites, were used as the template for real-time PCR analyses. (A) C_T values were plotted against log (amount of tachyzoites). (B) C_T values for all data points. Similar results were obtained in three independent experiments. NTC, no-template control.

sequence analysis confirmed the specific amplification of the B1 gene fragment (data not shown).

To assess the reproducibility and reliability of our real-time PCR assay, the B1 gene real-time PCR experiment was repeated four times under identical conditions. Each experiment was performed in quadruplicate. For the four experiments, the mean C_Ts were 25.02, 25.08, 25.32, and 25.13 and the intra-assay coefficients of variation (CVs) within each experiment (i.e., variations among the four sets of quadruplicates) were 0.40, 0.16, 0.24, and 0.79%. Accordingly, the mean C_T was 25.14 [(25.02 + 25.08 + 25.32 + 25.13)/4], and the mean interassay CV was 0.40% [(0.40% + 0.16% + 0.24% + 0.79%)/4].

To determine the detection limit of our method and to establish a standard curve that could be used for quantification, a serial dilution of *T. gondii* DNA with a final concentration from 5,000 to 0.05 tachyzoites was subjected to real-time PCR analysis. We were able to detect the B1 gene at a concentration as low as 0.05 tachyzoite (C_T = 39.34) in a 50-μl reaction volume (Fig. 3B). The standard curve showed a linear range across at least 6 logs of DNA concentrations with a correlation coefficient of 0.9988 (Fig. 3A).

We further assessed the ability of our real-time PCR to detect *T. gondii* infections in clinical specimens. Thirty paraffin-embedded fetal tissue sections were used for this study. DNA was isolated from these tissue sections using a Genom-

TABLE 1. Results of real-time and nested PCR detection of the *T. gondii* B1 gene in paraffin-embedded tissues

Sample no.	Result of nested PCR	Real-time PCR values	
		C _T	<i>T. gondii</i> quantity ^a
1	+	39.80	0.34
2	-	40.00	
3	+	37.23	1.78
4	+	38.91	0.60
5	-	40.00	
6	-	40.00	
7	+	35.00	7.49
8	-	40.00	
9	-	40.00	
10	-	40.00	
11	-	40.00	
12	+	34.17	12.79
13	-	40.00	
14	-	40.00	
15	-	40.00	
16	-	40.00	
17	+	33.67	17.65
18	-	40.00	
19	-	40.00	
20	-	40.00	
21	+	32.93	28.44
22	+	36.06	3.78
23	-	40.00	
24	-	40.00	
25	-	40.00	
26	-	40.00	
27	-	40.00	
28	-	40.00	
29	+	37.24	1.77
30	+	37.36	1.64

^a The quantities were expressed as the number of tachyzoites per tissue section.

icPrep cell and tissue DNA isolation kit. An amount of 1/10 of each DNA sample isolated from each tissue section was subjected to real-time PCR analysis. In this assay, an increase of fluorescent signal above a preset threshold within 40 PCR cycles was considered positive (i.e., C_T < 40). Of the 30 tissue sections we analyzed, 10 (33%) were positive, with C_Ts ranging from 32.03 to 39.80 (Table 1). These results were consistent with those obtained by the nested PCR (Table 1). Furthermore, the relative quantity of tachyzoites in each DNA sample was determined using the standard curve presented in Fig. 3A. The amount of tachyzoites among the positive samples varied from 0.34 (sample 1) to 28.44 (sample 21) per tissue section (Table 1).

DISCUSSION

Toxoplasmosis has emerged as a major cause of encephalitis in AIDS patients. Severe manifestations in these patients may include hemiparesis, seizures, visual impairment, confusion, and lethargy. Congenital toxoplasmosis also occurs in infants born to mothers who are infected during pregnancy. To prevent severe toxoplasmosis complications, early diagnosis and routine screening of patients early in the course of human immunodeficiency virus infection and before organ transplantation are warranted.

Currently the enzyme-linked immunosorbent assay for detecting IgM antibodies appears to be a reliable procedure for the diagnosis of acute *T. gondii* infections. However, this test is generally unsatisfactory for AIDS patients with latent or reac-

tivated infections because they fail to produce an IgM response or an increasing IgG titer. Several PCR-based techniques have been developed as alternative diagnostic measurements for *T. gondii* infection. These techniques make use of the most conserved gene sequences among different strains of *T. gondii* (8), including the B1 gene repetitive sequence, the P30 (SAG1) gene, and ribosomal DNA. The use of the B1 gene for *T. gondii* detection originated with Burg et al. in 1989 (2), who combined PCR amplification with Southern blotting to detect a specific B1 gene product. Since then, several variations of assays have been reported that have improved sensitivity or specificity. For example, Pujol-Rique et al. designed a one-tube heminested PCR method with a sensitivity equivalent to 0.1 parasite (24). Pelloux et al. designed a new set of PCR primers for *T. gondii* detection in amniotic fluid (22). In the present study, we have developed a real-time PCR-based B1 gene-specific TaqMan assay for quantitative detection of *T. gondii*. We have demonstrated that real-time PCR of the B1 gene is extremely sensitive (0.05 parasite/reaction) and highly reproducible (mean interassay CV of 0.4%). This method has also been applied for the analysis of clinical specimens, including whole blood and amniotic fluids (data not shown). Although both nested and real-time PCR are useful in the analysis of clinical specimens (Table 1) and may achieve similar levels of assay sensitivity, the major advantages of real-time PCR are its ability to quantify the infection load of a clinical specimen and its long linear range over at least 6 logs of DNA concentrations (Fig. 2). Quantification of infection load has been used to assess disease severity and treatment outcome in human immunodeficiency virus and hepatitis C virus infections (10). To date there have not been comprehensive studies relating this application to *T. gondii* infection. A preliminary report suggested that quantitative PCR is useful in the diagnosis of ocular toxoplasmosis (21). The quantitative analysis may also be useful in comparing different drug regimens and in determining the prognostic value of treatment. To quantify the amount of *T. gondii* tachyzoites, Lee et al. had developed competitive nested PCR (18). However, this method not only is labor-intensive but also provides only semiquantitative data, with a narrow linear range of 2 to 3 logs of DNA concentrations. Secondly, the potential PCR carryover associated with conventional PCR is usually avoided in real-time PCR, since the latter is performed in a closed-tube environment. Thirdly, it is much less labor-intensive, since there is no need for post-PCR handling, such as agarose gel electrophoresis of the PCR product. In our hands, it takes a mere 2.5 h to complete the analysis of 10 specimens, as opposed to approximately 6 h for nested PCR. Finally, the adaptability of real-time PCR to a high-throughput 96-well format should significantly reduce the overall time spent per sample in a clinical laboratory.

In summary, the real-time PCR-based method described in this study provides a rapid, sensitive, and quantitative way of detecting *T. gondii* in clinical specimens. Thus, this method may be suitable for routine screening of *T. gondii* infection in the clinical laboratory in conjunction with other diagnostic techniques, such as serological tests. This technique is particularly useful in screening AIDS patients, who usually fail to generate specific IgM or increased IgG titers. Future study is warranted to further explore the clinical value of this technique.

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