

# Performance of 2 Polymerization Protocols Targeting Cloned Toxoplasma Parasites

Nihal A. Hanafy<sup>1,2\*</sup>, Mohamed S. Badr<sup>3</sup>, Ghada M. Nasr<sup>4</sup>

<sup>1</sup>Department of Parasitology, Faculty of Medicine, Cairo University, Cairo, Egypt; <sup>2</sup>Department of Microbiology, International Medical School, Management and Science University, Shah Alam, Malaysia; <sup>3</sup>Department of Molecular Biology, Medical Research Centre, Faculty of Medicine, Ain Shams University, Cairo, Egypt; <sup>4</sup>Department of Molecular Diagnostics, Genetic Engineering and Biotechnology Research Institute, Sadat City University, Sadat City, Egypt

## Abstract

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**\*Correspondence:** Nihal A Hanafy. Department of Parasitology, Faculty of Medicine, Cairo University, Cairo, Egypt. E-mail: nihai\_ahmed@msu.edu.my

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**BACKGROUND:** *Toxoplasma gondii* is a common parasitic infection of humans. Infection is usually mild. Serious complications can occur in pregnant and immunocompromised patients.

**AIM:** The present study aims to investigate the performance of 2 different PCR protocols; real-time quantitative molecular assays (qPCR) and conventional molecular assays (cPCR), using 2 different sets of primers and by using cloned purified *Toxoplasma* genomic substances to be evaluated as reference samples.

**METHODS:** The target DNA was provided in 8 different quantities.

**RESULTS:** Amplification failure was reported only with the cPCR in samples of low concentrations using both primer sets. Quantitative PCR detected the 8 different dilutions of the purified *Toxoplasma gondii* using the 2 sets of primers while cPCR was sensitive to detect only 6 different dilutions.

**CONCLUSION:** Generally real-time quantitative molecular assays, is easy to use method compared to conventional PCR assay and produces more reliable results within only one hour time but still the possible application of qPCRs in routine diagnosis necessitates analysis of a large number of clinical samples in further studies to make the proper choice.

## Introduction

*Toxoplasma gondii* (*T. gondii*) is a known frequent cause of infection in a lot of warm-blooded animals including humans. This parasitic infection constantly infects between 15 and 85% of the world adult human population depending on geographical location. Fortunately, most cases of human infection are mild, but the severe disease may happen in immunocompromised individuals and congenitally infected fetuses in which serious complications may arise. Early diagnosis is crucial to start treatment that drastically reduces the extent of damage [1] [2]. Diagnosis of active infection using serology is often defective because reactivation of hidden infection is not all the time associated with changes in the levels of antibody, and evolution of latent or dormant toxoplasmosis is highly unpredictable.

Furthermore, IgM detection does not

necessarily specify recent infection [3] [4]. Parasite DNA detection by PCR was, therefore, considered in many types of research as a step forward to improve and accelerate toxoplasmosis diagnosis by performing different PCR protocols including quantitative real-time PCR (qPCR) which has been first reported by Bell & Ranford-Cartwright, [5]. These lately described real-time assays allow amplification and concurrent DNA detection in one hour [6] [7] [8]. However, there is no available in vitro diagnostic molecular technique for parasitic infection, unlike many bacterial and viral infections. In general, there are various factors affecting the outcome of PCR including the targeted DNA, the selected primers, the choice of reference control in addition to the optimisation of other reaction condition [9]. Over 25 diverse primer pairs have been used in various assays, most of them aiming for the repetitive 35-copy-number B1 gene [10].

Searching for optimal technique, this work aimed to investigate the performance of 2 different

PCR protocols; qPCR and conventional PCR (cPCR), using 2 different sets of primers and by using cloned purified *Toxoplasma* genomic substances to be evaluated as reference samples.

## Methods

A cloned purified *Toxoplasma* DNA (Roche Diagnostics) was prepared according to the manufacturer's recommendations. The target DNA was provided in 8 different quantities to yield from  $10^0$  copies/rxn to  $10^8$  copies/rxn of *Toxoplasma gondii* target molecules in 5  $\mu$ l once dissolved. Start with the lowest concentration, a hole through the sealing foil was punched, and 45  $\mu$ l PCR-grade water was added to each vial. The target DNA was then mixed by pipetting the solution up and down 10 times. For quantitative PCR, LightCycler FastStart DNA Master<sup>PLUS</sup> Hybridization Probes<sup>®</sup> Kit (Roche Diagnostics, Hoffmann-La Roche Ltd, USA) was used, applying Fluorescence Resonance Energy Transfer protocol (FRET PCR). For amplification, 5  $\mu$ l of DNA was used for each reaction mixture. The resulting PCR fragment of *T. gondii* was analysed using software data analysis version 3.5.3 which was implemented as stated in the LightCycler<sup>®</sup> instrument operator's manual. The reaction mixture (20  $\mu$ l; Master<sup>PLUS</sup> Hybridization Probes<sup>®</sup> kit; Roche Diagnostic) contained each primer 0.5  $\mu$ M, MgCl<sub>2</sub> 5 mM and 5  $\mu$ l template DNA. Capillaries were tightly closed, centrifuged at 500 g for 5s, and was then amplified in a LightCycler<sup>®</sup> instrument. Amplification for 50 cycles was performed: 5s denaturation at 95°C, 10s annealing at 61°C and 15s extensions at 72°C. The overall ramp rate was 20°C/s. One fluorescence reading for each sample was recorded at the extension step. Results were quantitatively demonstrated by determination of the revealing threshold or the crossing point (Cp), which distinct the cycle when the fluorescence of the used sample exceeded the baseline signal significantly. Samples were demonstrated as a fractional cycle number. The two sets of *Toxoplasma* primers were; a) the RE region (GenBank accession number AF146527), a repetitive sequence of 200-300 repeats in the *Toxoplasma* genome, giving an amplified fragment of 134 bp in conventional PCR; b) the commonly used B1 gene producing an amplified product of 432 bp. The adjusted conditions for conventional PCR (cPCR), were; 0.8  $\mu$ l of every primer (50 pmol/ $\mu$ l), 20 mmol/L dNTPs and 1.25 U recombinant Taq polymerase in 1  $\times$  PCR reaction buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl, 1.5 mmol/L MgCl<sub>2</sub>, 0.1% Triton  $\times$  100). The total volume prepared for the reaction was 50  $\mu$ l, including 5  $\mu$ l of the individual DNA samples. The Q cyler Quanta Biotech thermal cycler was used for the conventional amplification using: 94°C for 2 min, followed by 30 cycles of 94°C

for 1 min, 57°C for 2 min and 72°C for 3 min. For negative control, PCR mixture with no DNA and with DNase-free water was used to detect any cross-contamination.

## Results

Quantitative PCR detected the 8 different dilutions of the purified *Toxoplasma gondii* using the 2 sets of primers. Figure 1 shows the results of using the cloned *Toxoplasma* DNA, all concentrations from  $10^1$  to  $10^8$  were detected by qPCR. Quantitative genomic estimation of these positive samples in qPCR (Figure 1) ranged from  $1.7 \times 10^1$  to  $9 \times 10^{10}$ .

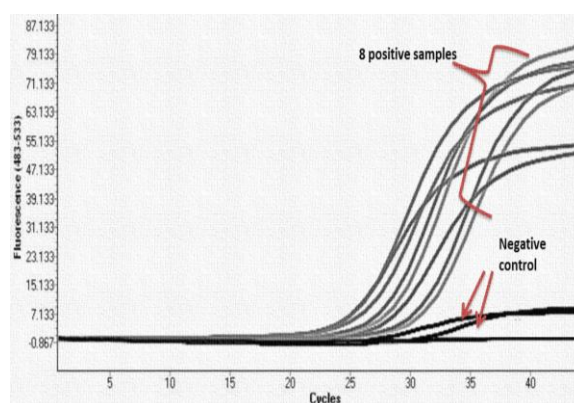


Figure 1: Eight positive samples of qPCR, starting fluorescence emission at different cycles and 2 negative control samples

Crossing points (Cps) showed different values ranged from 29.68 to 12.88 reflecting the different DNA quantities. Regarding cPCR, the amplified fragments were separated by gel electrophoresis at (432 bp for the B1 gene and 134 bp for RE region) as shown in Figure 2. From the 8 serially diluted purified samples included in the present study, conventional PCR was sensitive to detect  $10^3$  copies while failed to detect the low concentrations of ( $10^1$ ) and ( $10^2$ ) with both primers targeting B1 gene or RE region. No PCR contamination was observed during the study, as confirmed by the constant negativity of negative controls.

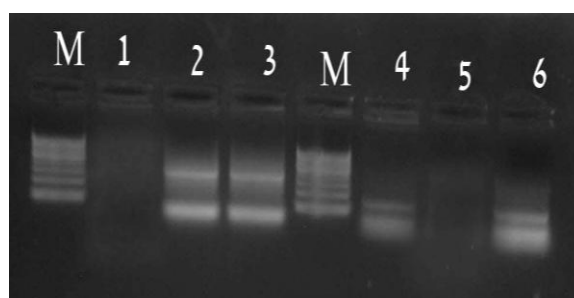


Figure 2: Lane 1 and 5 are negative control samples, M lanes are 100bp DNA markers. Lane 2 and 3 are the positive samples of the B1 gene (432bp), 4 and 6 positive results for RE region (143bp)

## Discussion

Almost one-third of humans have been exposed to *T. gondii* parasite. Latest estimates of the disease burden rank toxoplasmosis as a foodborne infection at the same level as salmonellosis or campylobacteriosis [1]. These increase the requirement of a sensitive quantitative diagnostic assay to check the severity of such parasitic infection in different clinical situations especially in high-risk groups. Lately, molecular techniques using LightCycler® devices have been greatly improved and supported by flexible software technologies which expand their implementation on unique analytic parameters to diagnose a wide range of infectious agents. No molecular kits are available for regular diagnosis of toxoplasmosis, but only for research purposes [6]. In the present study, amplification failure was reported only with the cPCR in samples of low concentrations using the both primer sets. In general, the main advantages of real-time qPCR over cPCR assays as mentioned by the previous authors are as follow; (a) it is less labor-intensive, the test is done in one step, while in cPCR at least two steps are needed; (b) it is performed in a closed system with no need for post-PCR treatment and therefore reduce the risk of any contamination (c) the result of the quantitative PCR (after DNA extraction) can be given in less than one hour for FRET-based assay versus a minimum of 6 hours with the conventional PCR [3] [11]. The LightCycler® device has additional advantages over the thermal cycler used in the conventional PCR, which is the quantitative evaluation of the products. Such numerical exploration of the parasite load may have prognostic implications during follow up after treatment of infected subjects and could thus verify the practicality in comparing different drug regimens. This was also reported in a review done by Lui et al., [11] concerning the different methods used for diagnosis of toxoplasmosis, he recorded that the real-time PCR can be used to evaluate the efficiency of treatment since the intensity of the infection can be estimated. As for all parasitic diseases, molecular diagnosis of toxoplasmosis is still not standardized. Due to the previously mentioned advantages of qPCR over conventional PCR, it seems highly possible that there will be an agreement on real-time PCR assays in the future. In a relatively recent study done by Santos et al., [12], qPCR was able to diagnose *T. gondii* in patients with uveitis, and they recorded the reliability of this assay in diagnosing toxoplasmic active focal necrotizing retinochoroiditis. However, such an agreement on the choice of the most suitable sequence to be amplified will be more challenging. The possible application of qPCRs in routine diagnosis necessitates analysis of a large number of clinical samples in further studies to make the proper choice. Certainly, the cost of the molecular assay—and the reagents needed to extract the DNA template beside the choice between the conventional

or qPCR techniques should be cautiously evaluated, excluding the cost of the PCR device instruments which may be bothering, however in the initial phase only

To conclude, real-time quantitative molecular assays are easy to use method compared to conventional PCR assay and produces results more readily. However, the chosen target gene can very much affect the results of the PCR assays, as we checked. Protocols for Real-time PCR should be optimised and properly evaluated in a larger number of samples before being implemented as routine diagnostic methods.

Nevertheless, these molecular tests certainly offer an alternative to conventional or even nested-PCR for a fast, easy and accurate diagnosis of toxoplasmosis. *Toxoplasma* B1 the tandem-arrayed 35-fold-repetitive gene is still a useful target which can be used for detection of *Toxoplasma* strains in clinical samples. Primers targeting RE region or other more repetitive genes seemed to be more sensitive. However, further comparative studies are recommended using a large number of suspected clinical samples to confirm variable sensitivities. Cloned *Toxoplasma* templates may facilitate easier and cleaner preparation of *Toxoplasma* reference samples especially when animal houses are defective in research or diagnostic laboratories.

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