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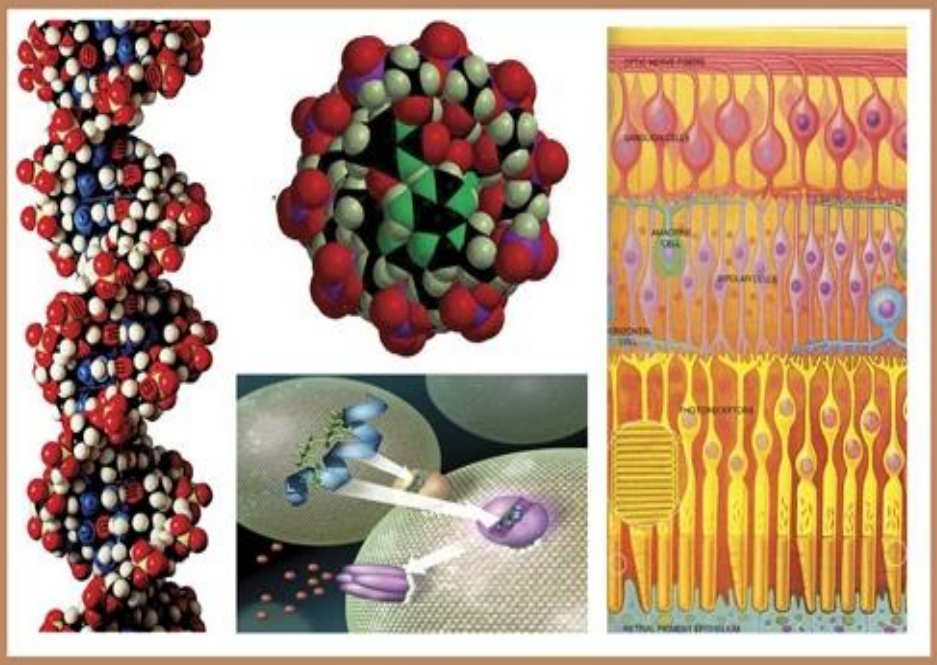

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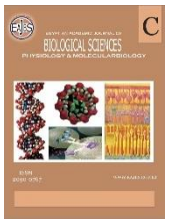

Rapid Detection of Viable *Toxoplasma gondii* by Nucleic Acid Sequence-Based Amplification (NASBA), Using Primer Sets Targeting B1 rRNA Gene

Yosra Nabil Abdel-Hafez[1], Asmaa Abd-Alghany [2], Mohamed S. Badr[3] and Marwa A. Elmallawany[2]

1-Department of Medical Parasitology, Faculty of Medicine, Fayoum University, Fayoum, Egypt
2-Department of Medical Parasitology, Faculty of Medicine, Cairo University, Egypt
3-Department of Molecular Biology, Medical Research Center, Faculty of Medicine, Ain Shams University

*E. Mail: mohamedsbadr@med.asu.ed

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ABSTRACT

Many diagnostic tests are currently used to diagnose chronic toxoplasmosis, yet the biggest challenge remains the rapid diagnosis of active infection, either primary or secondary. The late appearance of antibodies or their inability to emerge in case of defective immunity represents obstacles with serological testing. Molecular diagnosis relied on the detection of DNA are available, however, detecting DNA does not mean the presence of viable infectious organisms. Therefore, the switch to RNA detection technologies is necessary. In this initial study, a trial was done for early detecting genomic materials of viable circulating *Toxoplasma gondii* in the blood of 24 infected mice at variable doses, ranging from 10/ml to 10^8/ml *Toxoplasma gondii* RH strain tachyzoites, *applying* Real-time Nucleic acid sequence-based amplification technique (NASBA) and primer sets targeting B1 rRNA gene. The standard curve of real-time NASBA was created using serially diluted specific RNA samples that all generated signals for absolute genomic quantitation and crossing points ranging between 11.07 and 31.32. Fluorescence signals were created from all samples isolated from the 24 infected mice with different infection doses with quantitative genomic equivalent ranging from 1.3×10 to 9.6×10^{10} with crossing points ranging between 12.1 and 37.43, while no signals were detected from all negative control samples. In conclusion, NASBA is a relatively rapid primer-dependent molecular technique that can be performed in a single tube at a constant temperature for continuous amplification of a huge amount of nucleic acid. The technique can be used in medical diagnostics as an alternative to PCR, being quicker and sensitive to detect viable *Toxoplasma* circulating parasites.

INTRODUCTION

Toxoplasma parasite is one of the most widespread parasites in the world and almost one-third of the human population is currently infected. Fortunately, it does not affect the healthy immune cells, but it poses a great danger to certain vulnerable groups, such as those with weak immunity or pregnant women if they are infected for the first-time during pregnancy, exposing their pregnancy and fetuses to great risk (Fisch *et al*., 2019).

Although there are many diagnostic tests that help to detect chronic infection, the biggest challenge remains the rapid diagnosis of active infection, either primary or secondary, following tachyzoites transformation with much controversy about the ability of the current diagnostic tools to detect the circulating parasitic stages which necessitate rapid management to avoid life-threatening sequelae (Boyer *et al*., 2005). Diagnosis commonly relied on the serological testing that faces difficulties such as the late appearance of antibodies or their inability to appear in case of defective immunity (Calderaro *et al*., 2006). Perhaps with the advent of molecular technologies, some hopes appeared in obtaining a more favorable and sensitive diagnosis for detecting the circulating parasitic stages for rapid treatment, thus saving the vulnerable groups (Robert *et al*., 2021).

In general, molecular diagnosis relied on either detection of microbial deoxyribonucleic acid (DNA) or Ribonucleic acid (RNA). However, detecting DNA does not mean the presence of viable infectious organisms, which is a vital sign in the case of *Toxoplasma*, especially in high-risk groups. Therefore, the switch to RNA detection molecular technologies is necessary (Liu *et al*., 2015).

NASBA technique is a molecular tool that allows selective amplification of single-strand RNA within only 2 steps that takes RNA and helps to anneal specific primer sets, then amplifies the target gene using a specific enzyme cocktail (Deiman *et al*., 2002). The traditional PCR was initially intended for DNA molecular augmentation, yet PCR can amplify RNA, but with an additional reverse transcription step. Using the isothermal step as a usual phase in NASBA enables it to avoid amplification of homologous double-stranded DNA, thus evading the need for DNA-free RNA for NASBA reaction.

In this study, a trial was done too early to detect genomic materials of viable doses of circulating *Toxoplasma gondii* in the blood of infected mice, *applying* Real-time Nucleic acid sequence-based amplification technique and primer sets targeting B1 rRNA gene.

MATERIALS AND METHODS

Parasitic Stages:

Toxoplasma RH strain was kindly provided by *Schistosoma* Biological Supply Program (SBSP) at Theodor Bilharz Research Institute (Giza, Egypt) as well as the experimental animals. Following ethical and institutional guidelines, animals were maintained and infected by intraperitoneal injection. Following peritoneal lavage, parasitic stages were collected, washed and centrifuged twice by PBS at 2000 g for 10 min. Hemocytometer was used for counting the parasitic stages (Absher, 1973) and the samples were used for nucleic acid extraction. Using a 23-gauge needle, 24 mice were injected intraperitoneally, with various doses of *Toxoplasma* infection, from 10/ml to 10^8/ml of fresh *Toxoplasma gondii* RH strain tachyzoites (one dose for every 3 mice for a total of 24 mice). Then, the blood samples were collected 48 hours after initial inoculation. Nucleic acid was isolated from the conjunctive vein whole blood and then was stored at -70 °C till used for molecular investigation.

Molecular Technique:

According to the manufacturer's instruction, the High Pure RNA Isolation kit was used [Roche Life Science, Germany] was used for nucleic acid extraction. The yield of the nucleic acid was estimated (ng nucleic acid/ ml sample). For interpretation of purity of the extracted genomic materials, 260/280 ratio was calculated, aided by NanoDrop technology [Delaware, USA]. According to the method of Norouzi et al. (2016) for primer and transcription, the following primers and probes were used; forward primer T7 Toxo F; 5'-GGACTGGCAACCTGGTGTC-3', reverse primer T7 Toxo R; 5'AATTCTAATACGACTCACTATAGGGAGAAGGACCCGGACCGTTTAGCAG-3' and for molecular

beacon probe; 5'-FAM-cagcgACAGAAC AGCTGCAGTCCGGAAATACGCTG-3'. The latter was labeled with FAM fluorescent dye as well as non-fluorescent quencher DABCYL. Reaction was performed using a master mix gradient device [Eppendorf, Germany]. Reactions were applied using final volume of 20µl, involving 0.2 µM/primer and 0.1 µM of probe, 5 µl of RNA template. Incubation for 2 minutes was done at 65 °C and then at 41 °C for 2 minutes for uncoiling process. Then, 5 µl of enzyme-mix containing RNase H, T7 polymerase and AMV-reverse transcriptase was added and the final volume was then incubated at isothermal temperature 41 °C for 90 minutes to finalize amplification process. Fluorescence was measured every 120 s. RNA from *Toxoplasma gondii* strain RH was used as a positive control. RNase-free water was added to the NASBA reaction replacing the *Toxoplasma* genomic RNA template as negative controls. A standard curve was created from serially diluted parasites for absolute quantitative estimation and analytical investigation. The concentration of the samples was prepared to be from 10^{1} to 10^{8} genomic equivalent/ reaction.

RESULTS

The used method to extract the genomic materials achieved a good yield of nucleic acid, 86 ng/mg sample with a purity of 1.24-2.16. A standard curve of real-time NASBA was created using the serially diluted total RNA samples that all generate signals for absolute genomic quantitation (Fig. 1, A) and crossing points ranged between 11.07 and 31.32 (Fig. 1 B). Fluorescence signals were created from all samples isolated from the 24 infected mice with different infection doses (Fig. 1, C), after 48 hours of infection with quantitative genomic equivalent ranging from 1.3 × 10 to 9.6 × 10^{10} with crossing points ranging between 12.1 and 37.43, while no signals were detected from all negative control samples.

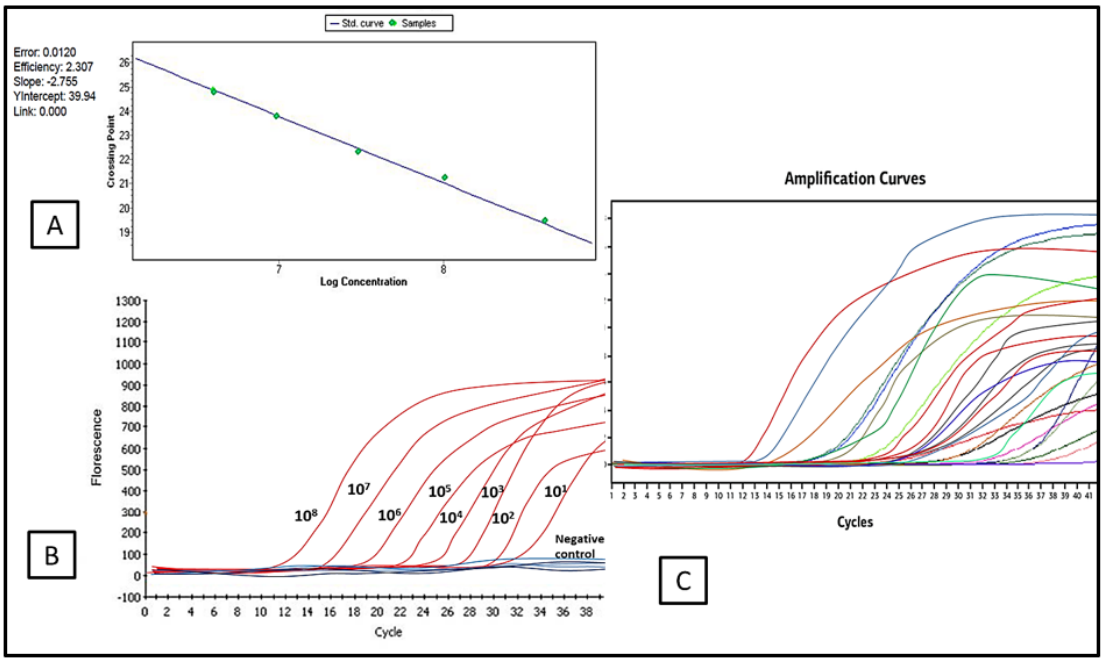

Fig. 1: A; Standard curve of real-time NASBA, showing different concentrations of the examined serially diluted samples. B; amplification curves related to the serially diluted *Toxoplasma* samples from 10 to 10^8 (red curves), while the blue straight lines represent the negative control samples. C; fluorescent signals created from the 24 samples of infected mice by the different parasitic loads.

DISCUSSION

Although *Toxoplasma* is one of the most widespread parasites in the world, dangers remain within specific groups, which are the weakest immunologically, and at that time, a quick diagnosis is very crucial to prevent serious complications. On many occasions, turn molecular testing becomes a choice to avoid obstacles facing some serological techniques. However, the diagnosis of parasitic viability remains a problem, not only within variable clinical samples that need viability confirmation but also in biological samples such as water samples (Marciano *et al*., 2020).

Lately, molecular diagnostic tools relied on the detection of genomic DNA and have proven to be more sensitive and specific than the traditional diagnostic methods. However, information about the viability of microbes cannot be achieved by standard DNA-targeted techniques such as PCR (Cangelosi and Meschke, 2014). Therefore, nucleic acid sequence-based amplification of RNA was actually developed by Compton (1991) for better diagnostic benefits. In this work, NASBA was performed to take single-stranded RNA related to *Toxoplasma gondii* and with the help of specific primers, annealing was achieved at 65℃, and then the genomic target was amplified at 41℃ to produce multiple copies of a single-stranded RNA, aided by an enzyme cocktail including. RNase H. RNA polymerase was applied to synthesize a complementary DNA strand, cDNA from the RNA genomic template when the primer was annealed. RNase H then degrades the RNA genomic template and the other primer binds to the cDNA to form double-stranded DNA, which RNA polymerase uses to synthesize copies of genomic RNA (Wand *et al*., 2018).

The technique was proved sensitive to detect early infection in all infected mice in this study with variable parasitic load, even an infection dose of 10 tachyzoites per mouse. This result is in agreement with Cornelissen *et al*. (2014), but the authors applied real-time PCR for DNA detection. However, the big difference related to NASBA remains that it can verify the viability of the parasitic stages, being RNA detection technique. Moreover, NASBA has several technical advantages, compared to the currently available diagnostic tests for RNA detection as reverse transcription RT-PCR. One of these benefits is the elimination of DNase treatment which is obligatory in RT-PCR to confirm the fluorescent signal that comes only from RNA amplification, in addition to its more rapidity (Klein and Juneja, 1997).

Comparison related to NASBA was done by many authors (Echevarria *et al*., 1998; Fan *et al*., 1998; Aguilar *et al*., 2000; Kehl *et al*., 2001 and Norouzi *et al*., 2016), in favor of NASBA. The technique has another advantage related to the constant, isothermal temperature that does not require a thermocycler, thus contributing to reducing material costs. NASBA reaction is so more competent than DNA techniques, which are limited to binary increases per cycle (Norouzi *et al*., 2016). In addition, the use of the beacon probe allows the detection of genomic materials in one tube, eliminating the use of gels or the subsequent image analysis as well as the risk of contamination (Echevarria *et al*., 1998).

In this study, using real-time NASBA assay, 24 samples were quantified within less than 4 hrs, compared to 16 hours which was previously recorded by Norouzi *et al*. (2016), when using RT-PCR. In conclusion, NASBA is a relatively rapid primer-dependent molecular technique that can be performed in a single tube at constant temperature for continuous amplification of a huge amount of nucleic acid. The technique can be used in medical diagnostics as an alternative to PCR that is quicker and more sensitive to detect viable *Toxoplasma* circulating parasites.

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