Original Article

Comparison of a Nucleic Acid Sequence-based Amplification (NASBA) and real-time reverse transcriptase PCR methods for detection of *Toxoplasma gondii* in rat blood samples

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Summary

The numbers of RNA amplification methods for detection of *Toxoplasma* spp. are increasing, however comparative studies on the performance of these different assays are lacking. The aim of this study was to compare two molecular assays for detection and quantification of *Toxoplasma* spp. in blood samples collected from experimentally infected rats. A set of specific primers and beacon probe were selected from the B1 rRNA gene of *Toxoplasma*. The assays using real-time detection proved to be both sensitive and specific. Nucleic acid sequence-based amplification (NASBA) method for detection of *Toxoplasma* spp. have advantages regarding sensitivity and potential quantitative population dynamics of *Toxoplasma gondii* in comparison with the RT-PCR method, but it is not often routinely used at present. NASBA had a detection limit of 1 parasite/ml of blood, while RT-PCR detected 10 parasites/ml. The results of real-time NASBA can be obtained 12h earlier. Therefore, sooner than the ordinary real-time RT-PCR the use of real-time NASBA is preferred to the ordinary real-time RT-PCR.

Keywords*: Toxoplasma gondii*, NASBA, Real-time RT-PCR, B1 rRNA gene, rat.

Introduction

Toxoplasma gondii is an obligatory intracellular organism that infects one-third of the world population and is an opportunistic pathogenic parasite, causing toxoplasmosis (Montoya and Liesenfeld, 2004). Toxoplasmosis generally occurs by ingestion of contaminated food on undercooked meat containing tissue cysts. Neonatal or acute infections in pregnant women can give rise to congenital mental retardation, blindness and hydrocephalus (Duval et al., 2004). In immuno-compromised hosts (i.e. acquired immunodeficiency syndrome(AIDS), organ-

transplanted and some cancer patients), the development of fatal *Toxoplasma* encephalitis is believed to be due to the transition of resting bradyzoites to rapidly replicating tachyzoites (Boyer et al., 2005). A rapid, accurate and effective diagnosis is crucial and desirable to initiate adequate treatment in these two populations.

Current diagnosis of toxoplasmosis relies on serological detection of specific anti-*Toxoplasma* immunoglobulins. The congenital toxoplasmosis of a fetus may remain undetected since the pregnant mother might serologically be negative during the active phase of *T. gondii* infection. Serological testing may fail during the active phase of *T. gondii* infection because the antibody titers. Moreover, serological tests may fail to detect *T. gondii* infection in certain immunocompromised patients due to the fact that the titers of anti-*Toxoplasma* antibodies may fail to rise (Calderaro et al., 2006). Therefore, molecular amplification techniques can provide highly sensitive and specific methods for the detection, identification and characterization of toxoplasmosis in these patients (Bretagne et al., 1993). However, due to the robustness of DNA, the signal is not necessarily related to the detection of viable infectious parasites. Therefore, there has recently been interest on the use of RNA as the molecular target for viability assessment. Both mRNA and rRNA have a shorter half-life than DNA, and therefore, they should provide a better indication of viable organisms than DNA. However, if mRNA is used there must be certainty of existence of targeted mRNA for transcription under most assay conditions (Sheridan et al., 1998; Birch et al., 2001).

NASBA, first described by Kievits et al (1991) involves the simultaneous use of three enzymes, avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase, under isothermal conditions (41 °C). The final amplification product is a single-stranded RNA of opposite sense of the original target. More recently, real-time detection based on molecular beacon probes has been described (Leone et al., 1998). Furthermore, nucleic acid sequence based assay (NASBA), has been developed for the diagnosis of various infectious parasites, including Plasmodium (Schoone et al., 2000), *Leishmania* (Meide Van der et al., 2005) and *Trypanosoma brucei* (Mugasa et al., 2008).

In the present study, the NASBA was adapted to real-time molecular beacon technology for diagnosis of toxoplasmosis based on their B1 rRNAs. Molecular beacons are small, single-stranded nucleic acid, hairpin probes that brightly fluoresce when they are bound to their targets (Tyagi and Kramer, 1996).

Materials and Methods

The RH strain tachyzoites were obtained after peritoneal lavage of mice inoculated with the RH strain. Parasites collected from the mouse ascetic fluid were washed and resuspended in phosphate- buffered saline, and then centrifuged and washed twice, with centrifugation at 2000 g for 10 min. The concentration of tachyzoites was determined by phase-contrast microscopy using the counting chamber. The parasite pellets were used for RNA extraction.

Nucleic acids were extracted by the High Pure RNA Isolation kit (RNxplus) from whole blood according to the manufacturer's recommendations. Nucleic acid concentration was determined by spectrophotometry using the NanoDrop ND-1000 device (NanoDrop Technologies, Delaware, USA) and the 260/280 and 260/230 ratios were calculated (Sambrook and Russell, 2001).

The oligonucleotide primers and probes used in this study are shown in Table 1. To the specific primers for *toxoplasma* were designed based on the conserved region of the B1 gene from GenBank.

Molecular beacon was designed for *toxoplasma* based on the probe sequence with additional sequences at either end to form the stem structure of the molecular beacon. The stability and predicted structure of the beacons were analyzed by using the European MFOLD server (http://bibiserv.-techfak.uni-bielefeld.de/mfold/). The 5' and 3'ends of the molecular beacons were labeled with the fluorescent dye fluorescein (FAM) and the nonfluorescent quencher, 4-(4'-dimethylaminophenylazo) - benzoic acid (DABCYL), respectively. Sequences of the primers and probe are given in Table 1.

The Real-time NASBA using was synthesized by *in vitro* transcription from a PCR product obtained by *T.gondii* genomic DNA and primers T7 ToxoF and ToxoR (Table 1). Reactions were performed with TaqMan PCR core reagents (Applied Biosystems-Roche Molecular Systems Inc, Branchburg, NJ, USA) and 50-µL final volume including 1× bufferII, 1.5 mM MgCl2, 200 µM dNTPs, 900 nM of each primer and 1 U AmpliTaq Gold DNA polymerase. Reactions were run in a Master Cycler Gradient device (Eppendorf AG, Hamburg, Germany), using the following program: 10 min at 95 °C; 40 cycles of 20 s at 95 °C, 30 s at 58 °C and 1 min at 72 °C; and 7 min at 72 °C. The DNA-Toxo-1730 product was quantified by spectrophotometry and analyzed by standard ethidium bromide-stained agarose gel electrophoresis (2% w/v).

Transcription was performed with 4µl 5X Buffer, 2µl DTT, 100mM, 20–40u Recombinant RNasin® Ribonuclease Inhibitor, 4µl rATP, rGTP and rUTP (2.5mM each) (prepared by mixing 1 volume deionized water with 1 volume of each of the 10mM rATP, rGTP and rUTP stocks supplied) 2.4µl 100µM rCTP (diluted from stock) 1µl linearized template DNA, 15–20u T7 RNA Polymerase.The reaction mixture is incubated at 37°C for 60 minutes RNA was analyzed by electrophoresis using denaturing conditions in an agarose/formaldehyde gel system.

Real-time NASBA on B1 rRNA *T.gondii* (GenBank accession number AF179871) was performed on a NucliSens EasyQ analyzer (bioMe´rieux) using the Nuclisens Basic kit for amplification according to the manufacturers manual at a KCl concentration of 80 mM. Reactions were carried out in a final volume of 20 µl. The reaction mixture was prepared by sequential addition of 10 µl of NASBA reagent primer mix containing 0.2 µM of each primer and 0.1 µM of the molecular probe, 5 µl of RNA template and 5 µl of enzyme-mix containing RNase H, T7 polymerase and AMV-reverse transcriptase. Before the addition of the enzyme mixture, the mixture was incubated at 65 °C for 2 min to uncoil secondary and tertiary structures,

and then held at 41 °C for 2 min. The final mixture was incubated at 41 °C for 90 min to complete the amplification. As a negative control for each run, RNase-free water added to the NASBA reaction instead of the *Toxoplasma* RNA template controls. RNA from *T. gondii* strain RH was used as positive control. Fluorescence was measured every 120 s.

The results were considered positive when a typical logarithmic curve above the threshold line was obtained.

The primers and probe for Real-time RT-PCR were based on the same B1 rRNA sequence as used for the Real-time NASBA (Table 1). Briefly, 5 µl of extracted or diluted RNA was reverse-transcribed using200 units of M-MLV-RT (Invitrogen Canada Inc.), 5 µM of pd (N) 6 random hexamers (Amersham Biosciences), 0.25mM of each dNTPs (Promega Corporation), 2mM DTT (Invitrogen Canada Inc.) and incubated at 25 ◦C for 10 min, 37 ◦C for 50 min, followed by 5 min at 94 ◦C to stop the reaction. Complementary DNAs were then stored at −20 ◦C until further use in a two-step conventional RT-PCR or TaqMan real-time RT-PCR. Conventional PCR amplifications were performed using the same 116-bp B1 rRNA sequence as used for the Real-time NASBA described in Table 1, using an Eppendorf Mastercycler gradient system (Brinkmann Instruments Canada, Ltd., Mississauga, ON, Canada). The reaction mixtures contained 1× PCR buffer with 1.5mM MgCl2, 0.20mM dNTP, and 0.20 µM of each primer, 1 unit of Platinum *Taq* DNA polymerase (Invitrogen Canada Inc.) and 1µl of cDNA in a 20µl reaction. Amplification conditions were: initial denaturation at 94 ◦C for 2 min, followed by 35 cycles of denaturation at 94 ◦C for 30 s, primer annealing at 58 ◦C for 30 s and extension reaction at 72 ◦C for 45 s. A final extension was performed at 72 ◦C for 5 min. The PCR products were then analyzed by electrophoresis on 3% agarose gels (Invitrogen Canada Inc).

Fifteen *Toxoplasma*-free rats were injected intraperitoneally using a 23-gauge needle, with 0.2 ml of 1×10^7/ml *T. gondii* tachyzoites of the RH strain, freshly collected from peritoneal lavage of mice. The optimum time to collect bloods is 48 hours after initial inoculation. RNA was isolated from conjunctive vein whole blood and was stored at -70 °C.

Table 1. Oligonucleotides used in the NASBA assays for *T.gondii*

Name	Type	Sequence	Position
ToxoF	Forward Primer	5'-GGACTGGCAACCTGGTGTC-3'	1730
ToxoR	Reverse Primer	5'-AATTCTAATACGACTCACTATAGGGAGAAGGA CCCGGACCGTTTAGCAG-3'	1823
ToxoMB	Molecular Beacon	5'-FAM-cagcgACAGAACAGCTGCAGTCCGGAAATAcgctg-DABCYL-3'	1749

Underlined: T7 promoter sequence.

Lowercase: stem sequences for molecular beacons.

Results

Studies of parasite, synthetic RNA and experimental samples confirmed the specificity of the *Toxoplasma* assays for their target sequence. In total, 15 blood samples from healthy controls and 15 blood samples from confirmed *T.gondii* cases were analyzed by real-time NASBA test. All healthy control samples were negative for the desired target.

To determine the sensitivity of the NASBA and RT-PCR combined with the molecular beacon, the same dilution series of purified parasite total RNA was used. (Fig.1 and 3).

Blood samples collected from rats were analyzed using the developed NASBA assays. In total, 15 blood samples from experimentally infected rats were identified as positive for *T.gondii* by real-time RT-PCR method (Fig. 2).

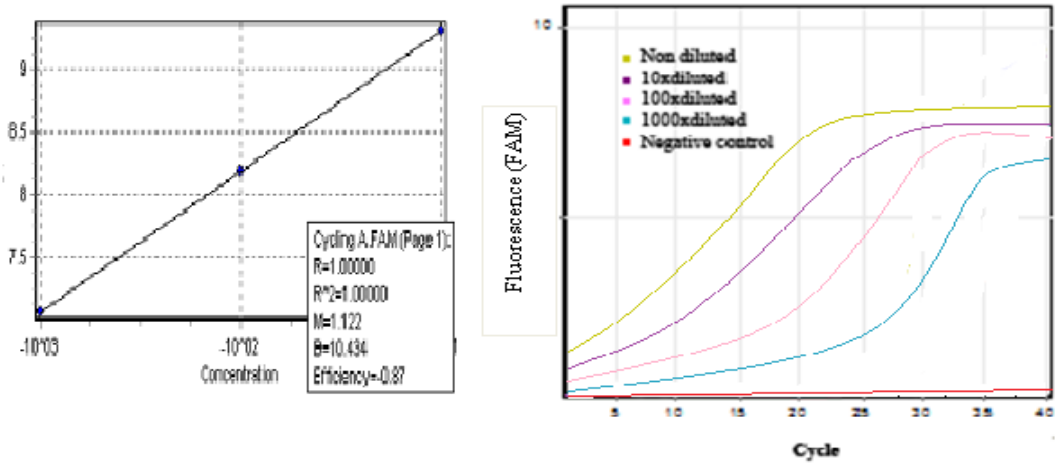

Fig 1. Standad curve of real-time NASBA amplification and detection by using the molecular beacon. Dilution series of total RNA from synthesized in vitro transcription and negative control consisted of total RNA from RNase-free water instead of the *Toxoplasma* RNA.

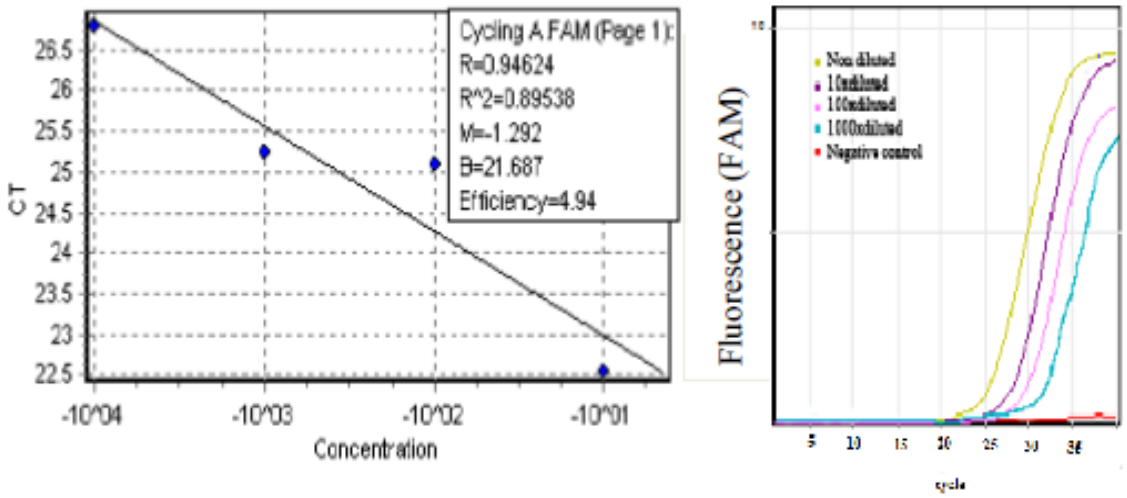

Fig 2. Standad curve of real-time RT-PCR amplification and detection by using the molecular beacon.

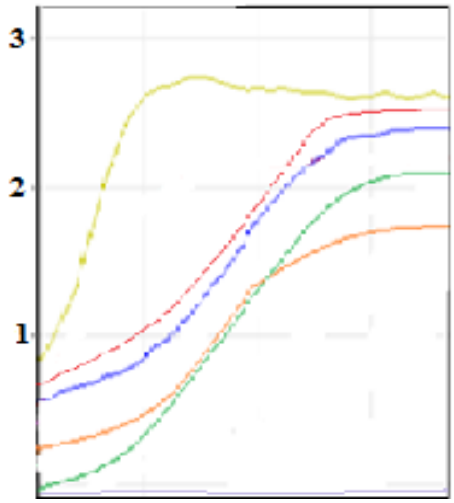

Fig 3. Real-time NASBA analysis of rat's blood samples artificially contaminated with *T.gondii.*

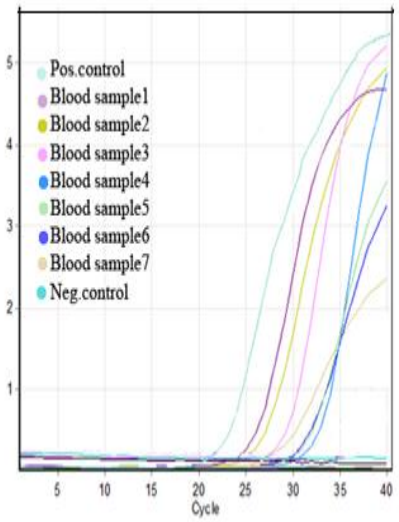

Fig 4. Real-time RT-PCR analysis of rat's blood samples artificially contaminated with *T.gondii.*

Discussion

Over the last decade diagnostic tests based on molecular biology have proven to be more sensitive and specific than the classical methods and thus chasing specific pathogens has greatly improved using these techniques. The literature showed that nucleic acid sequence-based amplification of RNA , developed for *toxoplasma* has several advantages when compared to the currently available diagnostic tools (ELISA and RT-PCR) (Klein and Juneja, 1997). This should eliminate the necessity for DNase treatment which is required when using RT-PCR to ensure that the signal comes only from amplification of RNA (Klein and Juneja,

1997). In addition to being more sensitive and more specific than these methods, hence being in a more reliable diagnostic, the assay is also easier and faster to perform than RT-PCR analysis. An advantage of the NASBA assay compared to the previously reported RT-PCR methods (Echevarria et al., 1998; Fan et al., 1998; Aguilar et al., 2000; Kehl et al., 2001) is the continuous, isothermal process that does not require a thermocycler. A constant temperature throughout the amplification reaction enables each step of the reaction to proceed when intermediate amplification becomes available.

Thus, the NASBA reaction is more efficient than DNA methods that are limited to binary increases per cycle (Stanway, 1990).

The application of the molecular beacons allows detection in a single tube, without the need of running gels and the use of membranes or image recording (Echevarria et al., 1998). This result in the reduction of labor, speeds up analysis procedure, and minimizes the risks of contamination and rules out errors during the process. In this study, developed a Real-time NASBA assay for the detection of *Toxoplasma gondii* adapted the potential use of the assay for analysis of clinical materials was investigated. Although this assay is useful for diagnosis, it is often expensive to establish in a routine diagnostic laboratories. The NASBA assay included a NucliSens Basic Kit, which provides the user flexibility over the targets to be tested. 'Real-time' detection is based on the use of molecular beacon probes in NASBA reactions generating a fluorescent signal for direct amplicon detection during the amplification process. RNA amplification with the generation of a target-specific fluorescent signal occurs simultaneously in a one-tube system and fluorescence is recorded by a fluorometer. Real-time monitoring of the NASBA reactions result in fluorescence plots over time with a typical initial exponential rate consistent with the cyclic phase of NASBA reaction. The latter is followed by a plateau phase, which represents the stage when the amplicon stops accumulating exponentially due to depletion of reaction components.

By the real-time NASBA assay, 48 samples were quantified within 4 hrs, comparing to real-time PCR 16hrs. Some other molecular tests had also been developed for the diagnosis of *T. gondii* (Costa et al., 2000). Real-time PCR assay has been used in the diagnosis of *toxoplasma* in Allogeneic Stem Cell. This PCR test gave reproducible quantitative results over a dynamic range of 0.75 parasites per PCR mixture, compared Real-time PCR assay and nested-PCR for the detection of *Toxoplasma gondii* (Adriana et al., 2006). Nested and real-time PCRs evaluated in this study that were very sensitive and specific assay.

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