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Original Article

Development of a Quantitative Real-Time PCR Assay for Detection of *Toxoplasma gondii* in Brain Samples

Mahboubeh Berizi [1], Jalal Babaie [1], Pezhman Fard-Esfahani [2], Marjan Enshaeieh [1,3], Rahmah Noordin [4], *Geita Saadatnia [3], *Majid Golkar [1]

1. *Department of Parasitology, Molecular Parasitology Laboratory, Pasteur Institute of Iran, Tehran, Iran*
2. *Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran*
3. *Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran*
4. *Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang, Malaysia*

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***Correspondence**
Email:
gitasaadat@gmail.com;
majid.golkar@gmail.com

Abstract

Background: Toxoplasmosis is a worldwide-distributed infection that can cause serious diseases, mainly in congenitally infected and immunodeficient individuals. PCR assays play an indispensable role in the detection of *Toxoplasma gondii* in different biological samples.

Methods: This study was conducted in the Parasitology Department at Pasteur Institute of Iran (Tehran) during 2016-2018. We designed a highly sensitive quantitative real-time PCR (RT-qPCR) targeted REP-529, a noncoding repetitive DNA. We cloned the amplicon in a plasmid (pTZREP-529) and used it to generate the standard curve. The Toxoplasma RT-qPCR characteristics, i.e., detection limit, specificity, linear dynamic range, linearity, intra-, and inter-assay precisions, were determined. The detection limit of the assay was one plasmid copy number (PCN) per reaction (about 0.004 *T. gondii* genome), and the linear dynamic range was equal to 6 logs (1×10^1 to 1×10^7 PCN per reaction).

Results: The assay showed no signal when genomic DNA of *Plasmodium falciparum*, *Leishmania major,* and *Trichomonas vaginallis* were used. The standard curve was drawn using dilutions of pTZREP-529 plasmid spiked with genomic DNA from a mouse brain, and test characteristics were shown unaffected. Applying the *Toxoplasma* RT-qPCR, we showed brain cysts were significantly decreased in mice vaccinated with GRA2 antigen of Toxoplasma formulated in Monophosphoryl Lipid A (MPL) adjuvant.

Conclusion: We have developed a quantitative, specific, and highly sensitive PCR for detecting *T. gondii* in biological samples.

Introduction

Toxoplasma gondii is a worldwide distributed intracellular protozoan parasite that is capable of infecting all warm-blooded animals including humans (1). Primary infection is usually asymptomatic in immunocompetent individuals and rarely associated with non-specific symptoms such as fever, lymphadenopathy, or retinochoroiditis. In contrast, primary or reactivated infection in immunocompromised patients may cause severe neurologic, ocular, or disseminated disease (2). Primary infection with *T. gondii* acquired during pregnancy can result in severe congenital disabilities like hydrocephalus, chorioretinitis, and even neonatal death. Cerebral toxoplasmosis is a consequence of reactivation of latent infection in immunocompromised patients, which is fatal if not diagnosed and treated soon (1, 3).

Diagnosis of *Toxoplasma* in several clinical situations, e.g., in congenital and reactivated toxoplasmosis, is confirmed by detecting parasite DNA. Real-time PCR is the most commonly applied method for this purpose, with very high sensitivity and specificity. Quantitative real-time PCR (RT-qPCR) can measure the parasite load in biological samples, thereby providing information about the severity of infection, its prognosis, and the necessity of drug dose adjustment (3, 4).

Most studies evaluated the efficacy of experimental *Toxoplasma* vaccines by increasing survival or decreasing brain cyst load in vaccinated animals (5-7). Quantification of parasite load in different organs, e.g., brain and spleen, provides further evidence regarding vaccination's protective efficacy. Microscopic examination of brain homogenate has commonly applied for cysts' enumeration, but it is tedious, labor-intensive, time-consuming, and subject to observation errors. Several PCR methods and target sequences have been developed and evaluated to detect *T. gondii* in different biological samples related to various clinical situations. The repetitive sequence REP-529 (Genbank accession No. AF146527), repeated between 200 to 300 times in the genome, is among the most used target sequences as it provided superior sensitivity in different clinical settings (8-10).

RT-qPCR can be used to measure the parasitic loads in biological samples, which may serve as a predictive tool for predicting the clinical course of the infection (11). Correlation between parasitic loads in amniotic fluid and severity of congenital infection was documented (12). Though more studies are required to confirm these correlations, RT-qPCR's accuracy also needs substantial improvements to obtain more homogenous results by different labs. The use of a common standard is an essential step toward more accurate RT-qPCR assays.

Most studies used purified genomic DNA of *T. gondii* as the standard for qPCR. The gDNA's significant drawbacks are possible variations in its quality over time, poor reproducibility, and stability. On the other hand, a plasmid DNA containing *T. gondii* DNA insert as a standard offers quality that is more consistent, reliability, reproducibility, and stability, thereby yielding more standardized RT-qPCR assays (13, 14).

We aimed to design and validate an RT-qPCR assay targeting the REP-529 sequence and develop a plasmid containing the REP-529 amplicon as the standard. The RT-qPCR characteristics, i.e., analytical sensitivity, specificity, linear dynamic range, linearity, intra-, and inter-assay precisions, were determined. Furthermore, we explored if the gDNA matrix of the mouse brain affects the amplification efficiency of RT-qPCR. This study's primary goal is to develop a quantitative, specific, and highly sensitive PCR for detecting T. gondii in biological samples.

Materials and Methods

This study was conducted in the Parasitology Department at Pasteur Institute of Iran (Tehran) during 2016-2018.

Parasite

Tehran strain of *T. gondii*, a type II strain isolated from a patient (15), was used in the study. The cysts were obtained from parasite bank of Parasitology Department of Pasteur Institute of Iran. NMRI mice were infected by intraperitoneal (i.p.) injection of 10 brain cysts, obtained from mice infected one month earlier, in 0.2 ml of phosphate buffer solution, PBS (16).

Animals

Female NMRI mice (20-25 g, Pasteur Institute of Iran) were housed in groups of 7 mice in standard polypropylene cages in a room with controlled temperature (23 ± 2.0 °C) and 12h light/dark cycle (6:00–18:00). They were fed ad libitum with rodents' chow and free access to drinking water. Animals were randomly divided into two experimental groups.

All experiments were conducted during the light phase according to guidelines of the Institutional Animals Ethics Committee of Pasteur Institute of Iran (Authorization code 93-0201-13085, 12 January 2015) and the European Communities Council Directive of 24 November 1986 (86/609/EEC) in a way to minimize the number of animals and suffering.

Mouse immunization

For immunization studies, two groups of mice (n=10) were subcutaneously immunized three times with two weeks intervals with 20 µg of recombinant GRA2 protein formulated in Monophosphoryl Lipid A (MPL) adjuvant, or with adjuvant alone. Three weeks after the immunization, mice were challenged with 10 Tehran cysts. One month later, mice were sacrificed, and their brain was stored at -80 °C until used.

Development of Quantitative Real-time PCR (RT-qPCR)

Design of primers and probe

A pair of specific primers were designed using AlleleID ver. 7.5, according to the published sequence of REP-529 (NCBI Nucleotide, accession No. AF146527). RT-qPCR was developed for the detection of REP-529. The expected size of the PCR products was 146 bp.

ToxoREP-F: 5'-TCTCGTCGCTTCCCAACCA-3'

ToxoREP-R: 5'-GTGCTTGGAGCCACAGAAGG-3'

Cloning of the amplicon in pTZ57R/T plasmid

Endpoint PCR was used using primer sets to obtain the PCR product of REP-529. The reaction mixture (20 µL) contained 10 µL of SmarTaq Master Mix (containing SmarTaq DNA polymerase, $MgCl_2$, dNTP, and 10X Buffer), 1 µL of the 10 µM forward and reverse primers, 1 µL of genomic DNA purified from Tehran brain cysts, and 8 µL of PCR grade water. PCR was carried out under the following conditions: an initial denaturation at 95 °C for 10 min, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 58 °C, and 30 sec at 72 °C. The final extension step was set for 20 min at 72 °C. The PCR amplicon was purified from agarose gel, cloned into pTZ57R/T plasmid, and transformed into *E. coli* JM107 (Bacterial strain #49759). Recombinant clones were selected by blue/white screening. Restriction analysis was performed to verify the insert in the recombinant plasmid. The insert's sequence was then determined, and one recombinant plasmid having the correct sequence of REP-529 amplicon (hereafter pTZREP-529) was used as the standard in RT-qPCR (17).

Construction of standard curves for plasmid copy number determination

The pTZREP-529 plasmid was used to generate standard dilution series and the development of the RT-qPCR test. The plasmid standard solution's concentration was measured using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA), and the corresponding copy number was calculated using online software (18). A ten-fold serial dilution series of pTZREP-529, ranging from 1×10^1 to 1×10^7 copies/µl, were made and used to construct the standard curve. The reaction mixture contained 1.0 µL of plasmid DNA dilution, 200 nmol/L ToxoREP-F, 200 nmol/L ToxoREP-R, 12.5 µL of RealQ PCR 2X Master Mix Green (Ampliqon), and PCR grade water to a final volume of 25 µL. An initial denaturation at 95 °C for 15 min, followed by 40 cycles of 10 sec at 95 °C and 15 sec at 61 °C. Threshold cycle (Ct) values in dilutions were measured in triplicate and plotted against their initial copy numbers' logarithm. Each standard curve was generated by linear regression of the plotted points, and the standard curve parameters were obtained (9, 19). The Ct was calculated under default settings for the Rotor-Gene 6000 system (Corbett Research, Australia) Software (ver. 1.7).

Standardization of the RT-qPCR

Each assay was designed to include negative and positive controls and the standard curve. The negative control was PCR reaction without template DNA. The positive control was a PCR reaction containing 7 ng gDNA of *T. gondii* Tehran strain. All controls and samples were assayed at least in triplicate. The specificity of *T. gondii* RT-qPCR was evaluated by performing the RT-qPCR using 200 to 2 ng of gDNA from *Leishmania major*, *Plasmodium falciparum*, and *Trichomonas Vaginalis*. The analytical sensitivity, i.e., the limit of detection, was established using 8 replicates of serially diluted pTZREP-529 plasmid at 10^7, 10^5, 10^3, 10^2, 10^1 and 1 copies/reaction. The RT-qPCR's inter-assay precision was defined as the coefficient of variation (CV) of Ct values obtained for each copy number/reaction in three different assays performed in three different days.

Effect of brain gDNA matrix on RT-qPCR

A serial dilution of RT-qPCR ranging from 1×10^1 to 1×10^7 copies/µl was used to generate the standard curve in the presence of 200 ng of gDNA purified from the brain homogenate of a mouse not infected with *T. gondii*. The real-time PCR protocol was performed as described above, and standard curve parameters were recorded.

Determination of parasite load in mice brain by quantitative RT-qPCR

The frozen brains were crushed into small pieces, were homogenized using a mortar and pestle, and passed several times through a syringe. Genomic DNA was purified using DNAeasy Blood & Tissue Kit (Qiagen) from 50 µL of each brain homogenate and used in RT-qPCR as the template to quantify REP-529 copy number in the brain of mice.

Statistical analysis

Data are presented as mean ± S.D. The REP-529 DNA levels are presented as a log unit. GraphPad Prism (ver. 7.5) was used for statistical analysis. If data had a normal distribution, they were analyzed by parametric test (independent samples t-test). Otherwise, the data were analyzed by nonparametric test (Mann Whitney U test). In all experiments, $P<0.05$ was considered statistically significant.

Results

Design of RT-qPCR for detection of T. gondii

To design a sensitive and specific RT-qPCR for detecting *T. gondii* in biological samples, a pair of specific primers were designed to amplify a 146 bp portion of the REP-529 sequence, 200 300 repeats in the genome of *T. gondii*. Instead of using *T. gondii* gDNA as the

reference standard for the standard curve generation, we chose to make a recombinant plasmid containing the amplicon (pTZREP-529). Quality characteristics were then evaluated toward developing a sensitive and reliable RT-qPCR to detect *T. gondii* in biological samples. The RT-qPCR was applied to determine parasite load in the brain of mice immunized with recombinant GRA2 and challenged with *T. gondii* cysts.

Construction of RT-qPCR plasmid

Gel agarose electrophoresis of PCR product showed a single band representing REP-529 (Fig. 1A). The amplicon was purified from the gel and cloned in pTZ57R/T plasmid. The size of the insert in the recombinant plasmid was confirmed by restriction analysis (Fig. 1B). The sequence of the insert was verified by sequence analysis of the recombinant plasmid (data not shown).

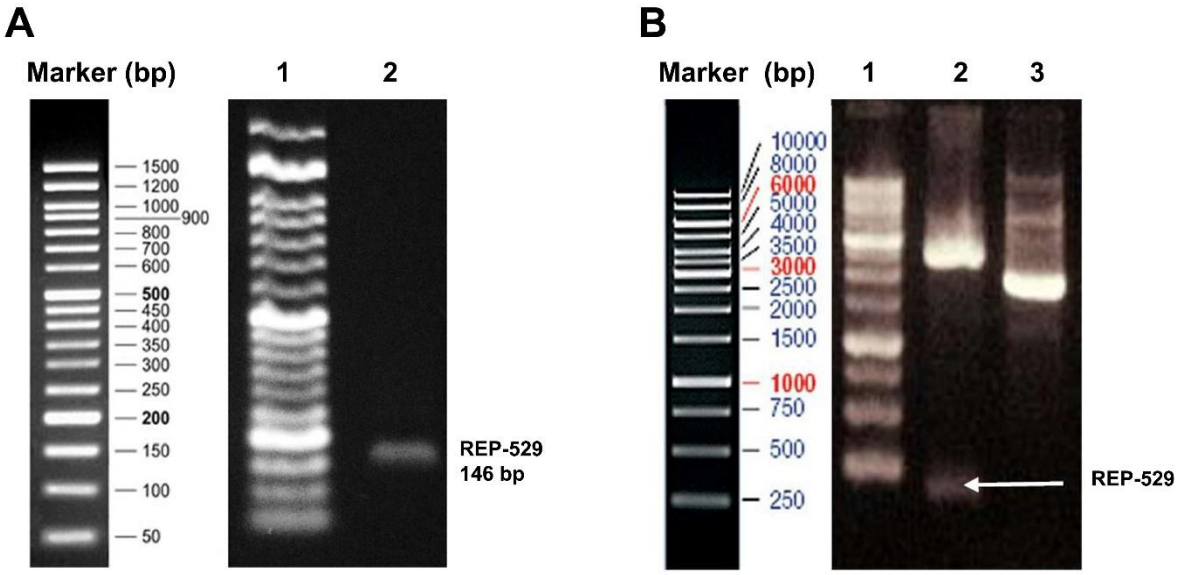

Fig. 1: The PCR product's agarose gel electrophoresis showed amplification of a DNA fragment sized 146 bp representing REP-529 DNA fragment (A). Agarose gel electrophoresis showed a DNA fragment sized about 146 bp, representing REP-529 DNA fragment, was removed from pTZREP-529 plasmid upon digestion with BamHI and *Eco*RI enzymes lane1. pTZREP-529 plasmid digested by *BamH*I/*Eco*RI, and lane 2 intact pTZ-REP-529 plasmid (B)

Development and Standardization of RT-qPCR for T. gondii

The RT-qPCR was performed using different copy numbers of the pTZREP-529 plasmid. (Fig. 2A). The linear dynamic range of the assay was 6 logs (Fig. 2B). The assay linearity was established (R^2>0.99718) between dilutions containing 10^1 to 10^7 copies/reaction. The standard curve slope was -3.046 which resulted in a high amplification efficiency of 113% (Fig. 2B). The results confirmed the assay's linearity at the defined range and the possibility of quantification of the target DNA in a wide range of the target concentrations. Intra-assay and inter-assay precision, presented, as the variation (CV) coefficient was less than 5% for different plasmid copies/reactions. The assay specificity was confirmed as no fluorogenic signal was detected when 200 or 2 ng gDNA of *L. major*, *P. falciparum* or *T. vaginalis*, three protozoan parasites with different evolutionary closeness with *T. gondii*, was used in the RT-qPCR (Table 1).

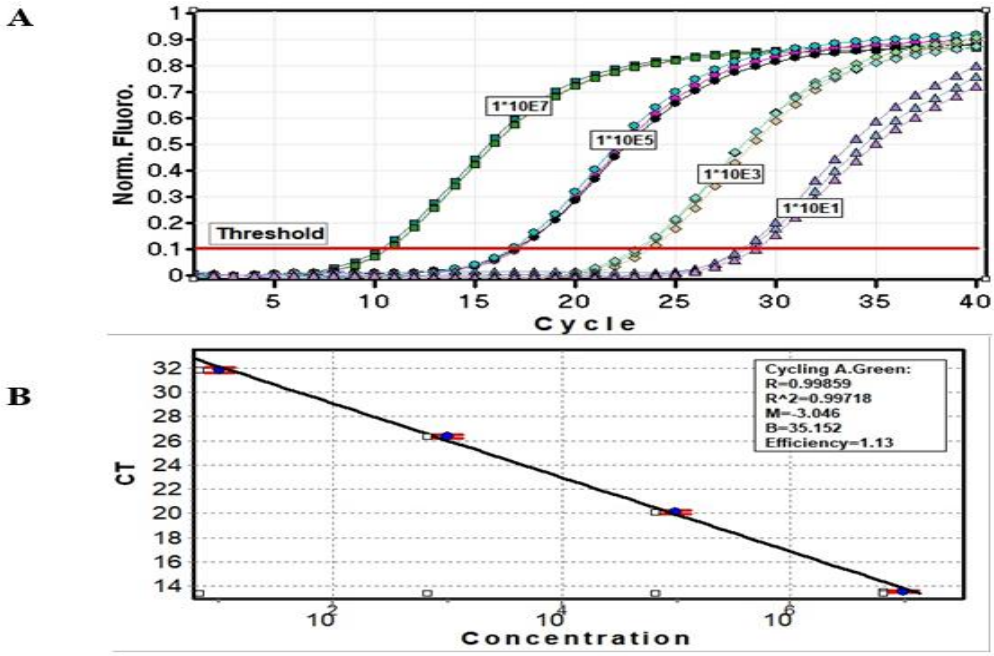

Fig. 2: Construction of the standard curve for quantification of the plasmid. The standard curve for the pTZREP-529 plasmid was generated using serial dilutions of the pTZREP-529 plasmid. C_t= -3.046*log (conc) +35.152

Table 1: Inter-assay precision of Toxoplasma RT-qPCR

Sample	*CT*	*Inter-assay precision*
pTZREP-529 10^7 copies/reaction	14.22	0.08
pTZREP-529 10^5 copies/reaction	20.88	0.01
pTZREP-529 10^3 copies/reaction	26.12	3.20
pTZREP-529 10^2 copies/reaction	30.40	0.35
pTZREP-529 10^1 copies/reaction	32.63	0.43

The assay limit of detection (LOD) was established as one copy/reaction, as 5 of 8 replicates gave a positive signal. However, the limit of quantification (LOQ) was 10 copies per reaction as the assay showed satisfactory linearity between 10^1 to 10^7 copies/reaction (Table 2).

Table 2: Determination of limit of detection and quantification for the Toxoplasma RT-qPCR

*Plasmid Copy No. **	*Ct mean*	*S.D.*	*No. of positive Reaction/No. Of rep-*
10^7	14.22	0.08	8/8
10^5	20.88	0.01	8/8
10^3	26.12	3.20	8/8
10^2	30.40	0.35	8/8
10^1	32.63	0.43	8/8 QL
1	33.89		5/8 DL

*(copies/reaction), Ct; Threshold Cycle, Q.L.; Quantification limit, DL; detection limit, S.D.; Standard deviation.

Evaluation of RT-qPCR performance in the presence of mouse brain gDNA matrix

To assess the matrix effect of the gDNA from mouse brain on the amplification efficiency, we performed RT-qPCR using pTZ-REP-529 plasmid, ranging from 10^1 to 10^7 copies/reaction, in the presence of 200 ng of gDNA purified from brain homogenate of a mouse not infected with *T. gondii*.

Interestingly, the assay performed equally in the presence of 200 ng of mouse brain gDNA, i.e., the linear dynamic range, linearity, and amplification efficiency were unaffected. Moreover, the Ct values of PCR reactions containing 10^1 to 10^7 copies of pTZREP-529 were not significantly different in the presence of 200 ng of gDNA from the mouse brain (P=0.6) (Table 3).

Table 3: Effect of gDNA on RT-qPCR CT values

*Plasmid Copy No. **	*Ct pTZ57R/T*	*Ct pTZ57R/T + gDNA*
10^7	14.22	14.52
10^5	20.88	21.09
10^3	26.12	27.62
10^2	30.40	31.27
10^1	32.63	33.47

*(copies/reaction)

Determination of parasite load in brains of mice using Toxoplasma RT-qPCR

We immunized two groups of mice with recombinant GRA2 formulated with MPL or with MPL alone. Immunized mice were challenged after the third immunization with cysts of Tehran strain. One month later, Genomic DNA was purified from brain homogenates of mice and used as the RT-qPCR template. The copy number of REP-529 DNA in each brain was obtained by multiplying the number at 4000 as dilution factor. The RT-qPCR results showed parasite load was decreased by 89% in immunized mice (P<0.05) (Fig. 3).

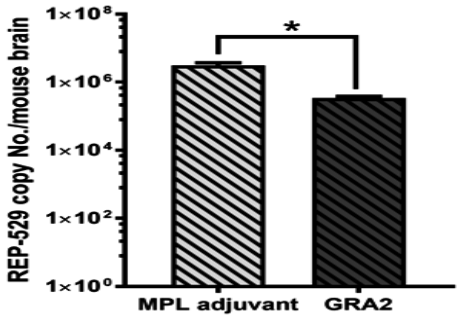

Fig. 3: Vaccination with GRA2+MPL decreased brain cysts in NMRI mice, as detected by Toxoplasma RT-qPCR. The intra-peritoneal route gave brain cysts; the results were expressed as mean± S.D. *P<0.05

Discussion

A standardized RT-qPCR is expected to provide sensitive, accurate and reproducible results. One essential requirement for developing a standardized PCR test is the availability of a common standard (calibrator) that is easy to produce in large amounts, stable, and shows consistent quality across different production batches. In this study, a plasmid DNA containing REP-529 sequence of *T. gondii*, pTZREP-529, was constructed and used as a calibrator to develop a reliable absolute quantitative RT-qPCR for quantifying parasite load in biological samples. The method was shown to be highly sensitive, accurate, and reproducible. The RT-qPCR method was successfully applied for quantifying parasite load in the brains of mice vaccinated with GRA2 antigen of *T. gondii*.

Early diagnosis of *T. gondii*, especially in high-risk patients, like immune-compromised transplant recipients, requires an accurate and sensitive method with the potential of DNA detection in low concentrations. Several real-time PCR analyses with various repetitive PCR

targets for sensitive detection of *T. gondii* have been performed, including the B1 gene with 35 copies in the parasite genome (8, 11, 20). However, REP-529 became highlighted when a low concentration of *T. gondii* DNA is present in a sample because this target is present in more copies than the B1 gene (8, 21). The minimum detection limit curve of both primers set, REP-529 and B1, was compared in a related study for detecting *T. gondii* DNA. REP-529 was more sensitive than B1 to detect one parasite in the sample against B1 limit detection of 100 parasites (11, 22).

A detection limit of 0.83 tachyzoites/ mL for Rep529 was reported using archived clinical intraocular specimens (23). In contrast, our assay's detection limit was one PCN per reaction, roughly equal to 0.004 *T. gondii* genome. The 80-Mbp genome equivalent of *T. gondii* equals about 80fg and the detection limit for 529bp repeat regions was 20fg (24). However, our results showed more sensitivity; these superior results may be obtained since we used plasmid in this study.

Replacement of standard endpoint PCR by RT-qPCR in industrialized countries was done by the year 2000 (20). Applying identifiable fluorescent probes gives this method some advantages, like minimizing contaminations and amplifying them by detecting PCR products in just a single step. RT-qPCR compared with nested PCR had similar or higher sensitivity and higher specificity because of inherent contamination risk associated with nested PCR (25). Significance of sensitive RT-qPCRs for therapeutic monitoring, adjusting therapeutic strategy has been confirmed. Interestingly, the presence of genomic DNA of amniotic sample in RT-qPCR reactions containing different copy numbers of the plasmid did not affect the Ct values or standard curve parameters (26).

Furthermore, the RT-qPCR can replace microscopic enumeration of brain cysts in evaluating protection afforded by immunization with recombinant GRA2. We have developed a sensitive and specific SYBR green RT-qPCR to detect *T. gondii* in biological samples. The test was capable of detecting one copy number of REP-529 sequence, approximately 0.004 tachyzoites per reaction. The test is also linear across 6 logs of parasite DNA concentration, making it suitable for parasite enumeration in clinical settings and experimental vaccination studies. The PCR assay showed a high R^2 value (0.99) and a slope at 3.38, corresponding to a PCR efficiency of 97.5%. These values are satisfactory for all three regression lines; the PCR assay is therefore linear, allowing correct quantification of the DNA target. Finally, the present study results suggested that REP-529 is a sensitive PCR target for the diagnosis of *T. gondii* DNA. A total combination of various parameters, including specificity, sensitivity, and detectability in the presence of host DNA and the target in all *T. gondii* isolates, makes the REP-529 a valuable target for further investigation q-PCR.

Conclusion

Real-time PCR can be used to diagnose toxoplasmosis in the clinical context, monitor the efficacy of treatment, and correlate parasitemia with clinical symptoms. Low concentrations of *T. gondii* DNA could be detected more sensitively and accurately by RT-qPCR using the 529-bp repeat element of *T. gondii* than when using the B1 gene. The procedures investigated are rapid and sensitive, and suitable for routine use. Early diagnosis of toxoplasmosis in immunocompromised-transplanted patients should significantly improve the rate of successful treatment. The 529-bp repeat element may become the preferred target when real-time PCR is used to diagnose toxoplasmosis. However, further work is required to ensure that this PCR target is conserved among different strains of *T. gondii.*

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Conflict of interest

The authors declare that there is no conflict of interest.

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