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Development of a Novel Protocol Based on Blood Clot to Improve the Sensitivity of qPCR Detection of *Toxoplasma gondii* in Peripheral Blood Specimens

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Abstract. Quantitative polymerase chain reaction (qPCR) for *Toxoplasma gondii* multicopy genes has emerged as a promising strategy for sensitive detection of parasite DNA. qPCR can be performed from blood samples, which are minimally invasive to collect. However, there is no consensus about what type of blood specimen yields the best sensitivity. The development of a novel protocol for qPCR detection of *T. gondii* using blood clot, involving an appropriate DNA extraction method and the use of an internal amplification control to monitor the reaction is presented in the current study. Assays directed to the *B1* and *REP529* genes were performed in spiked specimens of whole blood, guanidine–ethylenediaminetetraacetic acid blood, and clot. The clot-based qPCR was shown to be more sensitive when compared with other types of specimens, detecting five and 0.05 *T. gondii* genomes, using *B1* and *REP529* targets, respectively. Finally, a comparative analysis with samples from HIV patients with clinical suspicion of toxoplasmosis was performed, demonstrating the detection of four positive suspected cases with clots compared with only one using guanidine–ethylenediaminetetraacetic acid blood. The high analytical sensitivity and the cost-effective advantages offered by clot supports this methodology as a good laboratory tool to monitor parasite burden.

INTRODUCTION

Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii*, an obligate intracellular coccidian parasite that infects humans and virtually all warm-blooded organisms, including birds, livestock, and wild mammals.[1,2] The distribution of *T. gondii* is ubiquitous and the human interaction with this parasite is common; one-third of the human population is believed to be infected.[3,4]

In most cases toxoplasmosis is asymptomatic, however, it can cause serious and life-threading conditions in immunocompromised subjects.[3,5] Toxoplasma encephalitis (TE) due to reactivation of latent toxoplasmosis in HIV patients is the most severe clinical manifestation seen in this group.[6–8] In addition, the vertical transmission of this parasite is the most important among pathogenic protozoa in human pregnancy.[9] This makes toxoplasmosis an important public health concern and the reliability of precise detection of *T. gondii* is crucial for opportune diagnosis and further treatment.

Traditional diagnosis of toxoplasmosis includes serological tests. However, this kind of approach has low sensitivity because of reactivation of the infection is not always followed by changes in antibody production, and correlation with neuroimaging is needed for accurate diagnosis.[10–12] For this reason, direct demonstration of the presence of parasite in tissues or body fluids would be a breakthrough for the diagnosis of this disease.

In recent decades, a wide arrange of DNA-based detection assays have been developed for *T. gondii* detection. Among these, amplification of parasite DNA by quantitative polymerase chain reaction (qPCR) was performed in different specimens, including amniotic fluid, tissue samples, cerebrospinal fluid, and blood.[13–16] Based on the premise that the sensitivity of qPCR is enhanced by the number of target sequences, the *B1* gene (35 copies)[17] and the 529-bp repeat element (REP 529, more than 300 copies)[18] have been extensively used as targets for PCR detection.[16,19,20] Nevertheless, collection of some specimens, such as brain tissue or cerebrospinal fluid, represent a risk and are not always easily obtained. In this way, blood offers many advantages as a low-invasive specimen and its usefulness in the diagnosis of toxoplasmosis by qPCR have been widely reported.[16,21]

Detection of *T. gondii* by qPCR is usually performed with DNA obtained from whole blood[22–24] and some studies recommend mixing the fresh specimen with guanidine or ethylenediaminetetraacetic acid (EDTA) to prolong the lifespan of the sample before DNA isolation.[22] However, the shipping of this reagent is no longer allowed under the new International Air Transport regulations.[25] This is a great obstacle for sample transportation and the development of efficient methods for DNA extraction and therefore efficient qPCR protocols from other blood specimens are needed.

Although there is no consensus about what kind of blood specimen is optimal for qPCR analysis, blood clot offers many cost-effective advantages for the collection and transport because this specimen does not require special containers for storage. In addition, the usefulness of the clot in qPCR has been previously reported in the diagnosis of Chagas disease and invasive aspergillosis, showing increased sensitivity when

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compared with serum or other blood specimens.[25,26] However, clot has not been previously tested in the diagnosis of *T. gondii* and there is little information about the required parameters to maximize the recovery of parasite DNA from this specimen.

In this study, a practical method for appropriate DNA isolation from blood clot was developed for its use in qPCR directed to *T. gondii* genes *B1* and *REP529*. An internal amplification control (IAC) was used to monitor the reaction. Spiked samples were used to confirm the increased sensitivity of the clot-based qPCR compared with whole blood in EDTA and guanidine–EDTA blood (GEB) specimens. In addition, this strategy was applied in a comparative quantitative analysis among blood samples of HIV patients with neurological symptoms and clinical suspicion of toxoplasmosis. In summary, the technical usefulness of blood clot as a cost-effective specimen to improve the sensitivity of qPCR aimed to monitor *T. gondii* burden was demonstrated.

Methodology. *Ethical statement.* Patient collection protocols were approved by the institutional review boards of the study hospitals and associated institutions: Hospital Regional de Loreto, Iquitos, Peru; Hospital Nacional Dos de Mayo, Lima, Peru; Asociación Benéfica Prisma, Lima, Peru; Universidad Peruana Cayetano Heredia, Lima, Peru; and University of North Carolina at Chapel Hill, Chapel Hill, NC. All patients, or their health care proxy, provided written informed consent for the collection of samples and subsequent analysis.

Cell culture for parasite stocks. Monkey kidney fibroblast LLC-MK2 cells were cultured in Roswell Park Memorial Institute medium supplemented with 2% fetal bovine serum, ampicillin (1 µg/mL), and streptomycin (1 µg/mL) in a 25 cm^2 blue vented-cap plastic flask incubated at 37°C in 5% CO_2 and 95% humidity. Once the cell confluence was 70–80%, RH strain *T. gondii* tachyzoites were added (in a supplemented medium 100 mM sodium pyruvate, 15 g of L-glutamine and 500 g of essential amino acids) to the original cell culture. After 8–10 days, cultures were centrifuged and pellets were washed and resuspended in 1 mL of phosphate buffer saline 1×. Finally, the stock was diluted and counted in a Neubauer chamber until a desired load of 1×10^6 tachyzoites per mL was obtained.

Spiked blood specimen preparation. Five healthy donors between the ages of 20 and 30 years, seronegative to toxoplasmosis, and with no significant previous medical history were recruited. Blood samples were collected in tubes with EDTA and tubes without additives. Blood with EDTA was preserved at –80°C as whole blood. Blood with additives was centrifuged at 1,000 *g* for 15 minutes, after which serum was drawn off to obtain the clot. For GEB, blood within EDTA tubes was mixed with guanidine in a proportion of 1:1. Each type of sample from healthy donors was aliquoted in volumes of 1 mL and spiked with 1×10^6 tachyzoites (Figure 1).

Study participants and clinical samples. A total of 20 patients more than the age of 18, with ability to provide informed consent, who were previously diagnosed with HIV by two separate tests and with clinical suspicion of toxoplasmosis were recruited for this work. Ten individuals were recruited from Hospital Regional de Loreto, Iquitos, Peru (IQ-HIV), and 10 from Hospital Nacional Dos de Mayo, Lima, Peru (TOX-HIV). Participants from both locations were only included if their $CD4^+$ cells count was lower than 250 cells/mL and if they had an onset of neurological symptoms within the last

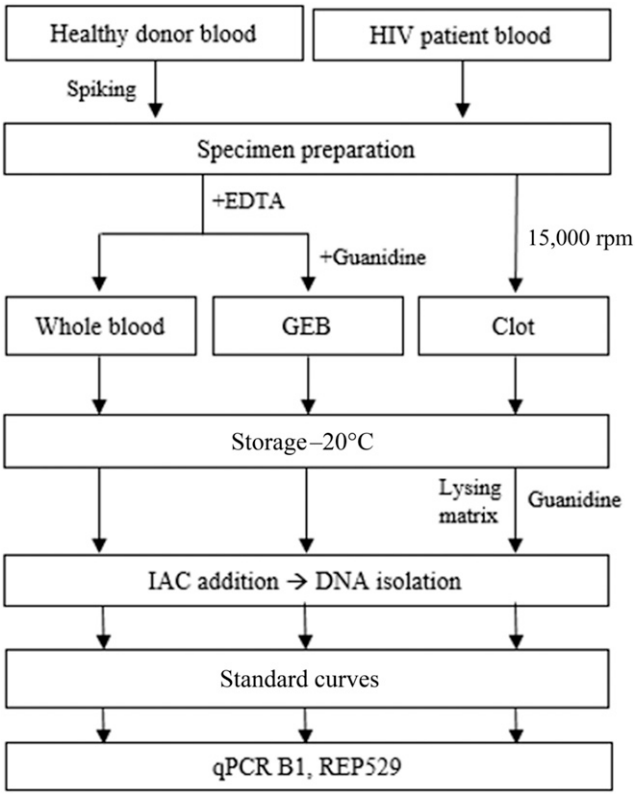

FIGURE 1. Schematic representation of study design. GEB = guanidine–EDTA blood; IAC = internal amplification control; qPCR = quantitative polymerase chain reaction.

6 months, or if they were examined by imaging and the diagnostic impression was associated with toxoplasmosis. This information was extracted from participants' charts and the comments of the neuroimaging studies. Peripheral blood was collected from each patient and clot and GEB specimens were prepared for analysis following the methods described previously.

Internal amplification control. To monitor the course of qPCR, a linearized plasmid containing a sequence of *Arabidopsis thaliana* (Table 1) was used as heterologous IAC.[27] Both spiked and clinical specimens were co-extracted with 40 pg of IAC added immediately before the DNA extraction.

DNA extraction. The specimens from both spiked and clinical samples were processed individually using High Pure PCR Template Preparation Kit (Roche Diagnostics Corp., Indianapolis, IN) following the manufacturer's instructions. For clot specimens we completed a preceding homogenization step involving the addition of 300 µL guanidine hydrochloride 6 M (Sigma-Aldrich). After mixing, the sample was transferred to a Lysing Matrix H 2 mL tube (MP Biomedicals, Santa Ana, CA) followed by an agitation cycle in a FastPrep-24™ 5G machine (MP Biomedicals) (5.5 m/second–30″) to ensure the clot disaggregation before the treatment with proteinase K. The DNA from each kind of sample was eluted with 100 µL of kit's elution buffer, quantified in NanoDrop™ (Thermo Fisher Scientific, Waltham, MA) and stored at –20°C. DNA obtained from spiked blood specimens was serially diluted 10-fold with healthy donor DNA solution to cover an expected range between 1 and 10^5 parasites for calibration curves.

TABLE 1
Primer sets and probe sequences used for the detection of *B1* and *REP529* by quantitative polymerase chain reaction in this work

Target	Probe/primer	Sequence
B1	Probe	FAM/BHQ 5′-CAA CAA CTG CTC TAG CG-3′
	Forward	5′-GCA TTG CCC GTC CAA ACT-3′
	Reverse	5′-AGA CTG TAC GGA ATG GAG ACG AA-3′
REP529	Probe	FAM/MGB 5′-AGG AGA GAT ATC AGG ACT GTA-3′
	Forward	5′-GCT CCT CCA GCC CGT CCA AAC T-3′
	Reverse	5′-TCC TCA CCC TCG CCT TCA T-3′
	Probe	VIC/MGB 5′-AGC ATC TGT TCT TGA AGG T-3′
Internal amplification control	Forward	5′-ACC GTC ATG GAA CAG CAC GTA-3′
	Reverse	5′-CTC CCG CAA ACC CTA TAA AT-3′

Serial dilution assay. To more effectively assess the limits of detection and analytical performance of qPCR assays, we calculated the parasite loads in terms of *T. gondii* genomes per PCR tube (Tgg) following the criteria described by Sterkers et al.[28] We reported the percentage of positive reactions after testing the qPCR protocol in five replicates for every spiked blood specimen in equivalents of 5, 0.5, and 0.05 Tgg per reaction.

Quantitative polymerase chain reaction detection of B1 and REP529. To perform a qPCR assay, fluorescence resonance energy transfer hybridization probes were used for genes *B1* and *REP529*. Both the probes and primers sequences have been previously tested for *T. gondii* diagnosis[29] (Table 1). A single qPCR included 10 µL Taqman® Universal PCR Master Mix II 1× (Roche), 1.2 µL of each primer (10 µM), 0.4 µL of probe (0.25 µM), 2.2 µL of MiliQ water, and 5 µL of DNA (20–150 ng/µL). In addition, 0.3 µL of IAC primers (5 µM) and 0.3 µL of probe (10 µM) were added. The amplification protocol involved two initial stages at 50°C and 95°C for 10 minutes each and 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute and was performed in a Light Cycler® (Applied Biosystems, Foster City, CA). The threshold cycle or cycle of quantification (Cq) obtained for each clinical sample was correlated with the calibration curve.

Statistical analysis. The experiments were performed in triplicates. For standard curve analysis, data were expressed as arithmetic mean ± standard deviation. Linear regression analysis was performed to test goodness of fit and Student's *t*-test was used to analyze the statistical significance of the observed slopes. A *P* value of less than 0.05 was considered statistically significant. All the tests were calculated using Prism software (Graphpad®, San Diego, CA).

RESULTS

Comparison of analytical sensitivity of qPCR from blood specimens. After the qPCR was performed, the quantification cycle (Cq) was obtained from the standard curves and the reportable range of *B1* and *REP529* between clot, whole blood, and GEB was compared. For this purpose, DNA isolated from spiked blood specimens were used to construct three independent standard curves ranging from 10^5 to 1 tachyzoites per mL. High linearity and adequate slopes were observed in curves from all samples, independent of the target. Calculated efficiency from all curves also showed high values (Table 2).

The Cq values obtained from the clot standard curve detected the lowest parasite load when compared with the ones from whole blood and GEB. Each dilution was evaluated in triplicate and the standard deviation obtained was less than 1.3. Assays with *B1* as target allowed for the detection of 10 parasites in clot as compared with 100 parasites in GEB and whole blood. By contrast, qPCR using *REP529* detected one parasite in clot as compared with 10 parasites in whole blood and GEB. Thus, there is a 10-fold increase in sensitivity when using clot for either *B1* or *REP529* assays (Figure 2).

However, the elution volume during DNA isolation might affect or overestimate the limits of detection of qPCR assay. To address this, we additionally performed a group of assays aimed to detect parasite load units in terms of 5, 0.5, and 0.05 Tgg. This choice allowed a straightforward comparison between specimens.

With *B1* as the target, the clot-based qPCR detected 0.5 Tgg in 60% of replicates, whereas assays in GEB and whole blood could only detect 5 Tgg properly. Again, when *REP529* is used as target, the qPCR performed in clot detected the lowest parasite load (0.05 Tgg) in all its replicates. By contrast, qPCR in whole blood (0.5 Tgg, 40% positives) and GEB (0.5 Tgg, 20% positives) showed a lower sensitivity and reliability (Table 3).

Detection of *T. gondii* in HIV patients' blood samples. To validate the standardized protocols, blood specimens from 20 HIV patients were analyzed by qPCR. Considering the higher sensitivity yielded using *REP529*, experiments in clinical

TABLE 2
Standard curve parameters from quantitative polymerase chain reaction assays performed for sample specimens

Specimen	Target	Slopes	Intercept	r^2	Amplification efficiency* (%)
Clot	*B1*	–3.449	42.59	0.975	94.95
	REP529	–3.400	35.72	0.994	96.84
Whole blood	*B1*	–3.259	39.33	0.994	102.64
	REP529	–3.332	29.56	0.98	99.99
Guanidine–EDTA blood	*B1*	–3.205	43.12	0.98	105.12
	REP529	–3.105	37.89	0.99	109.92

* Amplification efficiency was calculated based on the slope of the standard curve according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines.[34] In all the standard curves, the slope of the regression line is significantly different from zero ($P < 0.0001$).

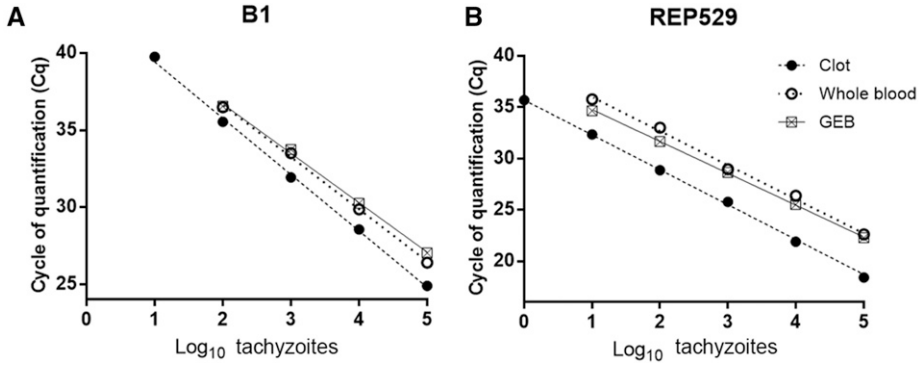

FIGURE 2. Comparison of dynamic ranges between distinct blood specimens for qPCR detection for *Toxoplasma gondii*. Standard curves were built with Cq obtained from three replicates for each dilution of DNA isolated from clot, whole blood, and guanidine–EDTA blood. Both targets, *B1* (**A**) and *REP529* (**B**), were tested for each kind of sample.

samples were performed only using this target. For each sample, only clot and GEB specimens were prepared simultaneously. Of the 20 patients, *T. gondii* was detected from clot specimens in four cases with the symptomatology of toxoplasmosis. By contrast, only one GEB sample was found to be positive (Table 4). These results reflect again, the high detection power of the clot samples over the other blood specimens. Whole blood was not analyzed because it was impossible to prepare immediately after collection.

DISCUSSION

The diagnosis of TE by PCR has been previously tested for HIV patients in cerebrospinal fluid and using the *B1* gene as the target, showing a sensitivity of 83%.[30] However, lumbar puncture is an invasive procedure that could be contraindicated in some cases. Therefore, the design of new protocols based on less invasive biological specimen collection represents a great opportunity to improve the power of DNA-based approaches for the diagnosis of toxoplasmosis. In the present study, we aimed to design an efficient protocol to detect *T. gondii* in blood clot by qPCR. Thus, the efficiency of distinct sample specimens was assessed for its practical usefulness in diagnosis. Two sets of primers and probes, directed to *B1* and *REP529*, were evaluated among the specimens.

After Cq analyses were performed, a better power of detection for all blood specimens was demonstrated when *REP529* was used as the target (Table 5). The effect of copy number in qPCR sensitivity has been discussed previously, and indicates the usefulness of *REP529* for the diagnosis of toxoplasmosis based on its high copy number and high level of conservation of nucleotide sequence between parasites isolates and strains.[16,18] Moreover, the heterogeneity of *B1* is well known and represents a great challenge for primer and probe design.[31] This fact may largely affect the amplification power because some parasite strains may not share the nucleotide sequences.

Although previous studies reported the low sensitivity of qPCR test based on blood for the diagnosis of TE,[32] the appearance of false negatives might be a consequence of the low recovery of DNA in the sample, the presence of PCR inhibitors, or the effect of large quantities of human genomic DNA competing with the parasite DNA. Therefore, the design of an appropriate test should include protocols that account for DNA-associated difficulties due to the nature of blood specimens.

Our results indicated that clot specimens yield a better sensitivity for qPCR when compared with whole blood and GEB (Figure 2, Table 5). The usefulness of clot as a diagnostic specimen has been discussed for Chagas disease where it was found to have a higher sensitivity compared with buffy coat and whole blood.[25] This phenomenon may be a consequence of parasite entrapment within the cellular portion of the clot or the sedimentation of circulating parasites. In addition, previous studies (H. Mayta, unpublished data) have shown that the sensitivity of qPCR is improved when the clot is treated with lysing matrices on a tissue homogenizer. These tools may allow a better lysis of the clotted blood and the trapped parasites, leading to a larger amount of DNA extraction. This observation is consistent with other reports for the diagnosis of invasive aspergillosis, where mechanical pretreatment of the clot increased DNA yield and improved the diagnostic sensitivity of the qPCR test.[26]

TABLE 3
Performances of qPCR for each blood specimen in terms of genomes of *Toxoplasma gondii* per qPCR

		50 Tgg		0.5 Tgg		0.05 Tgg	
Specimen	Target	Np/Total	%P	Np/Total	%P	Np/Total	%P
Clot	*B1*	5/5	*	3/5	60%	0/5	0%
	REP529	5/5	*	5/5	*	5/5	*
Guanidine–EDTA blood	*B1*	5/5	*	0/5	0%	0/5	0%
	REP529	5/5	*	1/5	20%	0/5	0%
Whole blood	*B1*	5/5	*	0/5	0%	0/5	0%
	REP529	5/5	*	2/5	40%	0/5	0%

qPCR = quantitative polymerase chain reaction; Tgg = *T. gondii* genomes. Quantitative polymerase chain reaction for each target was tested in five independent replicates to assess the number of positives from total (Np/Total) and the percentage of detection (%P). The parasite equivalents in Tgg were constructed considering 100 µL of elution volume during DNA extraction.
* 100% positive reactions.

TABLE 4
Quantitative polymerase chain reaction with blood samples from HIV patients with suspicion of toxoplasmosis using REP 529

Sample ID	Clinical impression	Quantitative polymerase chain reaction	Cq–clot	Cq–GEB
HIV patients				
IQ-004	Neurological syndromes. $CD4^+$ cells/mL: 73.	NEGATIVE	–	–
IQ-007	Neurological syndromes. $CD4^+$ cells/mL: 195.	POSITIVE	35.18 ± 0.21	–
IQ-008	Neurological syndromes.	NEGATIVE	–	–
IQ-009	Neurological syndromes. $CD4^+$ cells/mL: 30.	NEGATIVE	–	–
IQ-010	Neurological syndromes. $CD4^+$ cells/mL: 38.	NEGATIVE	–	–
IQ-012	MRI: Inflammatory process related to infectious disease, toxoplasmosis. Neurological syndromes. $CD4^+$ cells/mL: 45	POSITIVE	34.83 ± 0.18	–
IQ-013	CT: Multiple granulomas, brain strokes, and toxoplasmosis. Neurological syndromes. $CD4^+$ cells/mL: 33.	NEGATIVE	–	–
IQ-014	Neurological syndromes. $CD4^+$ cells/mL: 44	NEGATIVE	–	–
IQ-015	MRI: Brain strokes, toxoplasmosis. Neurological syndromes. $CD4^+$ cells/mL: 182.	NEGATIVE	–	–
IQ-023	MRI: Multiple granuloma, vasogenic cerebral oedema, and toxoplasmosis. Neurological syndromes. $CD4^+$ cells/mL: 97.	POSITIVE	32.59 ± 0.33	33.47 ± 0.51
TOX-02	Neurological syndromes. $CD4^+$ cells/mL: 97.	NEGATIVE		–
TOX-03	Neurological syndromes. $CD4^+$ cells/mL: 153.	NEGATIVE	–	–
TOX-04	MRI: Toxoplasmosis and progressive multifocal leukoencephalopathy. Neurological syndromes. $CD4^+$ cells/mL: 13.	NEGATIVE	–	–
TOX-05	Neurological syndromes. $CD4^+$ cells/mL: 82	NEGATIVE	–	–
TOX-06	Neurological syndromes.	NEGATIVE	–	–
TOX-07	MRI: Multiple granuloma, cerebral oedema, and toxoplasmosis. Neurological syndromes. $CD4^+$ cells/mL: 138.	NEGATIVE	–	–
TOX-08	Neurological syndromes.	NEGATIVE	–	–
TOX-09	MRI: Inflammatory process related to infectious disease and toxoplasmosis. Neurological syndromes. $CD4^+$ cells/mL: 45	NEGATIVE	–	–
TOX-10	MRI: Inflammatory process related to infectious disease, brain strokes, and toxoplasmosis. Neurological syndromes. $CD4^+$ cells/mL: 35.	POSITIVE	31.15 ± 0.37	–
TOX-11	MRI: Brain strokes and toxoplasmosis. Neurological syndromes. $CD4^+$ cells/mL: 51.	NEGATIVE	–	–
Healthy donors				
H-01	Not significant.	NEGATIVE	–	–
H-02	Not significant.	NEGATIVE	–	–
H-03	Not significant.	NEGATIVE	–	–
H-04	Not significant.	NEGATIVE	–	–
H-05	Not significant.	NEGATIVE	–	–

Cq = cycle of quantification; MRI = magnetic resonance imaging; CT = computed tomography scan.

The diagnosis of toxoplasmosis is a difficult task and several studies have reported poor sensitivities in conventional PCR methods made from peripheral blood, probably as a consequence of low parasite loads.[33] These observations are congruent with our results, where the Cq for positive samples reached high values that reflect very low parasite load (Table 4).

The protocol designed in our study highlights the power of detection by clot as a reliable specimen for the diagnosis of toxoplasma in immunocompromised patients. Finally, our results corroborate previous studies on qPCR-based diagnosis of toxoplasmosis, supporting the use of this design for an efficient detection of parasite burden in blood samples. The analysis based on clot provides several advantages as a cost-effective sample. Moreover, the high sensitivity of clot qPCR suggests the widespread use of this type of specimen as a powerful resource for *T. gondii* detection.

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TABLE 5

Mean Cq of 10-fold dilutions of *Toxoplasma gondii* tachyzoites on blood specimens for quantitative polymerase chain reaction assays

	Mean Cq ± standard deviation					
	Clot		Whole blood		Guanidine–EDTA blood	
Tachyzoites/mL	*B1*	*REP529*	*B1*	*REP529*	*B1*	*REP529*
10^5	24.89 ± 1.12 (24.28–26.58)	18.23 ± 0.54 (17.69–18.77)	26.41 ± 0.31 (26.10–26.73)	22.62 ± 0.04 (22.58–22.67)	27.04 ± 0.29 (26.73–27.33)	22.28 ± 0.21 (22.09–22.51
10^4	28.55 ± 1.12 (27.88–30.23)	21.74 ± 0.56 (21.30–22.38)	28.87 ± 0.38 (29.48–30.25)	26.38 ± 0.14 (26.22–26.50)	30.25 ± 0.33 29.93–30.60	25.53 ± 0.19 (25.30–25.66)
10^3	31.94 ± 0.88 (31.24–33.23)	25.52 ± 0.14 (25.38–25.66)	33.51 ± 0.32 (33.21–33.86)	28.97 ± 0.20 (28.73–29.09)	33.74 ± 0.13 (33.66–33.90)	28.65 ± 0.38 (28.42–29.09)
10^2	35.54 ± 0.41 (35.15–36.11)	28.84 ± 0.12 (28.69–28.92)	36.49 ± 0.43 (36.17–36.98)	31.95 ± 0.31 (31.72–32.17)	36.56 ± 1.08 (35.64–37.76)	31.66 ± 0.38 (31.23–31.98)
10	39.22 ± 0.55* (38.83–39.61)	32.56 ± 0.39 (32.11–32.82)	NA	35.77 ± 0.21* (35.62– 35.92)	NA	34.65†
1	NA	36.01 ± 1.29 (35.09–36.93)	NA	NA	NA	NA

Cq = cycle of quantification; NA = no amplification.
* Two of three replicates detected.
† One of three replicates detected.

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