The Journal of Molecular Diagnostics, Vol. 19, No. 5, September 2017

the Journal of Molecular Diagnostics
jmd.amjpathol.org

Comparative Study and Analytical Verification of PCR Methods for the Diagnosis of Congenital Chagas Disease

Carolina I. Cura,* Juan C. Ramírez,† Marcelo Rodríguez,‡ Constanza Lopez-Albízu,* Lucía Irazu,‡ Karenina Scollo,* and Sergio Sosa-Estani*

From the National Institute of Parasitology "Dr. Mario Fatala Chaben", and the National Institute of Infectious Diseases,‡ National Laboratories and Health Institutes Administration "Dr. Carlos G. Malbrán", Buenos Aires; and the Institute of Investigation in Genetic Engineering and Molecular Biology "Dr. Héctor N. Torres",† Buenos Aires, Argentina*

Accepted for publication May 3, 2017.

Address correspondence to Carolina I. Cura, Ph.D., Av. Paseo Colón 568, C1063ACS Buenos Aires, Argentina. E-mail: cura.carolina@gmail.com.

Congenital infection is currently the first cause of new cases of Chagas disease in Argentina and nonendemic areas worldwide. Its diagnosis is of utmost importance to guarantee curative treatment. To improve such diagnosis, a transfer process of PCR tests to the national laboratory network has been initiated. We performed a comparative study of four PCR assays [two end-point PCR and two duplex real-time quantitative PCR (qPCR) procedures] to detect *Trypanosoma cruzi* DNA in blood samples. Because satellite DNA and kinetoplastid DNA qPCR methods showed the best performance and the use of two different molecular targets for confirmatory purposes has been recommended, these methods were selected to perform the transfer process and, in consequence, subjected to an analytical verification protocol based on international guidelines. The anticipated reportable range was verified between 0.25 and 10^5 parasite equivalents per milliliter of blood (par. eq./mL) for both qPCR methods, and the limit of detection was estimated to be 0.87 (95% CI, 0.62−1.24) and 0.43 (95% CI, 0.32−0.59) par. eq./mL for satellite DNA and kinetoplastid DNA qPCR methods, respectively. In addition, both qPCR methods showed trueness and verified precision in the highest and the lowest concentrations tested. This work provides critical knowledge of the technology transfer process planned to cover laboratories of the regional network with known installed facilities. *(J Mol Diagn 2017, 19: 673−681; http://dx.doi.org/10.1016/j.jmoldx.2017.05.010)*

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi*, remains a major public health concern in 21 Latin American countries, where close to 5.7 million people are infected, 25 million are at risk, and 7000 deaths are recorded annually.[1,2] *T. cruzi* is primarily transmitted by Triatomine insect vectors, blood transfusion, organ transplant, congenital infection, and oral transmission. The reduction of vector-borne and blood transfusion as the main ways of transmission has provided the opportunity to direct efforts to control other modes of CD transmission, such as congenital transmission. The World Health Organization estimated that there are 1.1 million women of childbearing age infected by *T. cruzi* and that the incidence of congenital CD is 8668 cases per year in Latin America[1] and 2000 in North America.[3]

It has been estimated that congenital infection occurs on average in 5% of children born to chronically infected mothers in endemic areas, with variations depending on the region.[4,5] In Argentina, the risk of congenital transmission has been estimated to be between 2.6% and 17%, the latter occurring in Las Lomitas, Formosa, an area that is hyperendemic for *T. cruzi* infection, with a prevalence of 17.5% in the general population.[4,6] In the whole country,

Supported by National Laboratories and Health Institutes Administration "Dr. Carlos G. Malbrán."

C.I.C. and J.C.R. contributed equally to this work.

Disclosures: None declared.

This article has been published under the Hinari Access to Research for Health program.

Copyright © 2017 American Society for Investigative Pathology and the Association for Molecular Pathology. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jmoldx.2017.05.010

approximately 28,000 infants are at risk for acquiring congenital CD and between 1000 and 1300 infants are expected to be born infected every year.[7]

Because congenital infection with *T. cruzi* is mostly asymptomatic, the infection may progress to severe chronic CD later in life, but it can be effectively treated within the first years of life if accurately diagnosed.[5] During the acute phase of CD, the level of parasitemia is high, and the parasite may be detected in the bloodstream. In contrast, the chronic phase is characterized by low parasitemia, and the diagnosis is normally achieved with serologic tests that can detect circulating anti−*T. cruzi* antibodies.[8,9] The use of at least two independent serologic tests is recommended for the clinical diagnosis of CD,[10,11] whereas the detection of *T. cruzi* DNA by PCR could be useful in some settings, such as i) acute CD, including cases of congenital transmission; ii) posttreatment follow-up of patients; and iii) diagnosis of CD reactivation in immunosuppressed patients.[12]

The current diagnostic algorithm for congenital CD requires that newborns be diagnosed by parasite microscopic detection in the first 8 to 10 months of life and by serologic methods after this period, which is when maternal antibodies are cleared from the infant's bloodstream.[13] However, the low sensitivity of parasitologic methods combined with issues associated with no complaisance for the serologic follow-up after the first 10 months of life have contributed to a large number of misdiagnosed cases, hampering the treatment of approximately 50% to 70% of children with congenital CD.[14,15] PCR methods seem to be more adequate for the diagnosis of congenital CD because of their high sensitivity and have been reported to be specific for the detection of *T. cruzi* DNA in blood samples from newborns,[16−20] demonstrating their use as a predictive tool in the diagnosis of congenital infection.[21] Several conventional end-point (cPCR) and real-time quantitative PCR (qPCR) amplification procedures to detect *T. cruzi* in human blood samples have been evaluated and standardized in an international multicenter study.[12] Recently, two duplex qPCR strategies based on TaqMan probes, designed to quantify both *T. cruzi* DNA and an internal amplification control in a single reaction tube, were subjected to an extensive analytical validation protocol and clinical evaluation with blood samples from patients with CD from different regions.[22,23]

The Instituto Nacional de Parasitología Dr. Mario Fatala Chaben, Buenos Aires, Argentina, a reference health care center of CD, has initiated a technology transfer process of molecular methods for the diagnosis of congenital CD to the Public Health System. Our aim was to compare the performance of available PCR procedures used to detect *T. cruzi* DNA in blood samples with the purpose of choosing the most suitable PCR method to be implemented in the transfer process. The methods selected were then subjected to an analytical verification protocol based on international guidelines.

Materials and Methods

Patients and Blood Samples

Ninety-eight guanidine-EDTA-blood (GEB) samples were used: 43 from patients (26 adults and 17 infants) with CD confirmed by at least two independent serologic tests (enzyme-linked immunosorbent assay, indirect immunofluorescence assay, and indirect hemagglutination assay)[15] and 55 from controls (25 adults and 30 infants) with nonreactive serologic test results. All GEB samples from infants were submitted after 10 months of age (mean ± SD age, 13.2 ± 2.6 months), when diagnosis was confirmed by serologic methods. Among samples from adults (mean ± SD age, 37.8 ± 18.4 years), those obtained from patients with reactive serologic test results, confirmed in at least three different samples, corresponded to pretransplant and posttransplant ($n = 16$) and pretreatment ($n = 10$) samples. On the other hand, seronegative samples corresponded to possible triatomine bite ($n = 23$) and work accident ($n = 2$) cases in which negative parasite microscopic detection and nonreactive serologic test results were confirmed after three consecutive controls performed every 30 days. Blood samples collected from adults and infants (5 and 0.5 mL, respectively) were mixed with an equal volume of guanidine hydrochloride (6 mol/L) and EDTA (0.2 mol/L) (pH 8.00) buffer (GE) and kept at room temperature for 48 to 72 hours to obtain the GEB samples. The GEB samples obtained from adults were boiled for 15 minutes and then kept at 4°C together with the GEB samples from infants (which were not boiled) until DNA extraction.

All samples belonged to patients that came to the Instituto Nacional de Parasitología Dr. Mario Fatala Chaben for CD diagnosis, were preexistent at the time this study took place, and were anonymized before being analyzed.

DNA Extraction

DNA was extracted from 300 μL of GEB samples using the High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) as described by Duffy et al.[22] Two hundred picograms of linearized internal amplification control was added to each sample before DNA extraction.[24] Extracted DNA was stored at −20°C until use in PCR analysis.

PCR Procedures

qPCR Procedures

Two duplex qPCR procedures were evaluated: satellite DNA (SatDNA) qPCR and kinetoplastid DNA (kDNA) qPCRs. The former targets the satellite sequence from *T. cruzi* nuclear DNA and the internal amplification control sequence.[22] The latter is a modification of the method described by Ramírez et al[23] and targets the conserved region of *T. cruzi* kDNA and the human *RNase P* gene, as an

endogenous amplification control, using the TaqMan RNase P Control Reagents Kit (Applied Biosystems, Foster City, CA) at a final concentration of 0.5× (Table 1). Both reactions were performed with 5 μL of extracted DNA, using FastStart Universal Probe Master Mix (Roche Diagnostics GmbH) in a final volume of 20 μL in an ABI7500 device (Applied Biosystems). A sample was considered positive for *T. cruzi* DNA when the amplification curve crossed the established threshold, resulting in a threshold cycle (C_T) value.

End-Point PCR Procedures

Two cPCR methods were performed as described by Velázquez et al[21]: one targeting a 188-bp sequence from SatDNA and the other one targeting the 330-bp variable sequence of kDNA (Table 1). In addition, the amplification of a fragment of the *ACTB* gene was used to ensure the quality of DNA sample (Table 1).[21] Amplifications were performed in a LifeTouch thermal cycler (BIOER, Hangzhou, China), and PCR products were visualized under a UV transilluminator after agarose gel electrophoresis using 2% agarose gels that were stained with ethidium bromide.

PCR Quality Controls

A negative control and two positive controls that contained 10 and 1 fg/μL of *T. cruzi* DNA were included in every run, as recommended.[25,26]

Comparative Study of PCR Methods

A retrospective diagnostic evaluation was performed using GEB samples from patients and the four PCR assays to detect *T. cruzi* DNA described above.

Statistical Analysis

StatisPro software (Analyse-it Software Ltd. and CLSI) was used to estimate the diagnostic parameters of the PCR assays, using serologic testing as the criterion for diagnostic accuracy. A three-way comparison between the two PCR methods (test and comparative methods) and the diagnostic accuracy criterion was used to estimate the 95% CIs for the difference between sensitivities and for the difference between the specificities of the PCR methods, as recommended in the CLSI EP12-A2 guideline.[27] If the calculated interval did not include 0, it was considered that there were significant differences between the PCR methods for the parameter being evaluated.

Analytical Verification of the Performance of the qPCR Assays

Analytical verification was performed for the SatDNA and kDNA qPCR methods, which have been extensively validated.[22,23] PCR reagents and conditions were the same as described above, with slight modifications: uracil-DNA glycosylase (UNG, Thermo Fisher Scientific, Rockford, IL) was added to the reaction mix at a final concentration of 0.005 U/μL to avoid carryover contamination, and the human *RNase P* gene was used as the internal amplification control in both qPCR methods.

The FastStart Universal Probe Master Mix (Roche Diagnostics GmbH) used in the original assays contains dUTP instead of dTTP, allowing its use in the UNG-modified methods. Original cycling conditions were modified with the addition of an initial 2-minute hold step at 50°C. To provide empirical evidence that neither such modifications nor the current laboratory conditions significantly changed the analytical performance of both qPCRs, the anticipated reportable range, the limit of detection (LOD), and the precision and trueness were verified as described below.

Spiked Blood Samples

Seronegative human blood samples were spiked with cultured epimastigotes of *T. cruzi* CL-Brener stocks and mixed with an equal volume of GE. The number of parasites was determined using a hemocytometer and verified in a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter Inc., Fullerton, CA).

Verification of the Anticipated Reportable Range

Ten milliliters of a seronegative human blood sample was spiked with 10^6 *T. cruzi* cultured epimastigotes and mixed with an equal volume of GE. This GEB sample, which contained 10^5 parasite equivalents per milliliter of blood (par. eq./mL), was serially diluted with noninfected human GEB to obtain a panel of GEB samples (10 mL each) with 10^4, 10^3, 10^2, 10, 1, 0.5, and 0.25 par. eq./mL (assigned values). DNA was extracted from 300 μL of each GEB dilution as described above for patient samples, and each extract was amplified in triplicate. Absolute quantification of parasitic loads was performed using a standard calibration curve (measured values). The standard curve was generated with serial dilutions of total DNA obtained from the 10^5—par. eq./mL sample, using negative DNA extracted from noninfected human GEB as diluent.[22,23] Assigned versus measured values were converted to $\log_{10}$ par. eq./10 mL of blood and plotted for linear regression analysis using SigmaPlot version 10.0 (SPSS, Chicago, IL).[25,28]

Verification of the LOD

A replicate test was performed to verify the reported LOD [the lowest parasitic load that gives 95% of positive results ($LOD_{95\%}$)] for both qPCR assays according to ISO/WD 16140-3.[29] Six DNA replicates from spiked GEB samples that contained 0.25 and 0.7 par. eq./mL (reported $LOD_{95\%}$ for the kDNA and SatDNA qPCRs, respectively)[22,23] and 1 par. eq./mL were extracted and amplified for four consecutive days (24 replicates in total for each dilution). Concentrations of 0.25 and 0.7 par. eq./mL were assayed using the kDNA qPCR and 0.7 and 1 par. eq./mL were assayed using the SatDNA qPCR.

Table 1 Sequences and Concentrations of Primers and Probes Used in the Comparative Study of PCR Methods

Method	Target	Oligonucleotide	Sequence	Final concentration, μmol/L	Reference
SatDNA qPCR	*Trypanosoma cruzi* SatDNA	Cruzi 1	5′-ASTCGGCTGATCGTTTTCGA-3′	0.75	22
		Cruzi 2	5′-AATTCCTCCAAGCAGCGGATA-3′	0.75	
		Cruzi 3 (probe)	FAM-5′-CACACACTGGACACCAA-3′-NFQ-MGB	0.05	
	IAC	IAC Fw	5′-ACCGTCATGGAACAGCACGTA-3′	0.1	
		IAC Rv	5′-CTCCCGCAACAAACCCTATAAAT-3′	0.1	
		IAC Tq (probe)	VIC-5′-AGCATCTGTTCTTGAAGGT-3′-NFQ-MGB	0.05	
kDNA qPCR	*T. cruzi* kDNA	32F	5′-TTTGGGAGGGGCGTTCA-3′	0.4	23
		148R	5′-ATATTACACCAACCCCAATCGAA-3′	0.4	
		71P (probe)	FAM-5′-CAT+CTCA+CC+CGTA+CATT-3′-BHQ1	0.05	
	Human *RNaseP*	TaqMan RNase P Control Reagents Kit (Applied Biosystems)		0.5×	
SatDNA cPCR	*T. cruzi* SatDNA	Tcz 1	5′-CGAGCTCTTGCCCACACGGTGCT-3′	2.0	21
		Tcz 2	5′-CCTCCAAGCAGCGGATAGTTCAGG-3′	2.0	
kDNA cPCR	*T. cruzi* kDNA	121	5′-AAATAATGTACGGGKGAGATGCATGA-3′	2.0	
		122	5′-GGTTCGATTGGGGTTGGGTAATATA-3′	2.0	
ACTB cPCR	*ACTB*	β Act F	5′-CGGAACCGCTCATTGCC-3′	2.0	
		β Act R	5′-ACCCACACTGTGCCCATTA-3′	2.0	

The + in front of the nucleotide indicates a locked nucleic acid monomer substitution.

BHQ, black hole quencher; cPCR, end-point PCR; IAC, internal amplification control; kDNA, kinetoplastid DNA; MGB, minor groove binder; NFQ, nonfluorescent quencher; qPCR, real-time quantitative PCR; SatDNA, satellite DNA.

The LOD of the modified qPCR assays was estimated using the PODLOD.xls version 8 application (Freie University, Berlin, Germany; *http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html*, last accessed January 13, 2017),[30] including the results of 24 additional replicates that contained 0.125 par. eq./mL that were assayed using kDNA qPCR and those obtained for the 15 replicates that contained 0.5, 10, and 1000 par. eq./mL from the precision verification experiments described below, as follows: results obtained for replicates that contained 0.125 (×24), 0.25 (×24), 0.5 (×15), 0.7 (×24), 1 (×24), 10 (×15), and 1000 (×15) par. eq./mL were used to estimate the LOD of the kDNA qPCR, whereas 0.5 (×15), 0.7 (×24), 1 (×24), 10 (×15), and 1000 (×15) par. eq./mL results were used to estimate the LOD of the SatDNA qPCR.

Verification of the Precision and Trueness

The precision and trueness were verified under the provisions of CLSI EP15-A2 guidelines.[31] Three replicates of each concentration of spiked GEB samples (0.5, 10, and 1000 par. eq./mL for the SatDNA qPCR, and 0.25, 10, and 1000 par. eq./mL for the kDNA qPCR) were tested for 5 consecutive days (15 replicates in total for each concentration). StatisPro software spreadsheets version 3.02.2 (Analyse-it Software Ltd. and CLSI, Wayne, PA) were used to calculate the repeatability (or within-run precision) (Sr)

Table 2 Comparison of the Performance of qPCR and cPCR Assays to Detect *Trypanosoma cruzi* SatDNA and kDNA in Blood Samples

Method	Target	Group	Total, *N*	TP, *n*	FP, *n*	TN, *n*	FN, *n*	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, %*	NPV, %†	Efficiency, %‡
qPCR	SatDNA	Total	98	26	2	53	17	60 (46–74)	96 (88–99)	93	76	81
		Infants	47	13	1	29	4	76 (53–90)	97 (83–99)	93	88	89
		Adults	51	13	1	24	13	50 (32–68)	96 (80–99)	93	65	73
	kDNA	Total	98	29	11	44	14	67 (53–80)	80 (68–88)	73	76	74
		Infants	47	13	7	23	4	76 (53–90)	77 (59–88)	65	85	77
		Adults	51	16	4	21	10	62 (43–78)	84 (65–94)	80	68	73
cPCR	SatDNA	Total	98	17	0	55	26	40 (26–54)	100 (93–100)	100	68	73
		Infants	47	12	0	30	5	71 (47–87)	100 (89–100)	100	86	89
		Adults	51	5	0	25	21	19 (9–38)	100 (87–100)	100	54	59
	kDNA	Total	98	25	4	51	18	58 (43–72)	93 (83–97)	86	74	78
		Infants	47	13	4	26	4	76 (53–90)	87 (70–95)	76	87	83
		Adults	51	12	0	25	14	46 (29–65)	100 (87–100)	100	64	73

*The proportion of subjects with a positive test result who actually have the target condition [100 × TP/(TP + FP)].

†The proportion of individuals with a negative test result who are free of the target condition [100 × TN/(TN + FN)].

‡The proportion of true results [100 × (TP + TN)/(TP + FP + TN + FN)].[32]

cPCR, end-point PCR; FN, false-negative results; FP, false-positive results; kDNA, kinetoplastid DNA; NPV, negative predictive value; qPCR, real-time quantitative PCR; SatDNA, satellite DNA; PPV, positive predictive value; TN, true-negative results; TP, true-positive results.

Table 3 Three-Way Comparison of PCR Methods

Comparison	Group	Difference, % (95% CI)	
		Sensitivity	Specificity
SatDNA qPCR vs kDNA qPCR	Total	−7 (−15.7 to 1.9)	16.4 (6.0 to 28.3)*
	Infants	0.0 (−14.4 to 14.3)	20.0 (3.4 to 37.5)*
	Adults	−11.5 (−24.6 to 2.5)	12.0 (−4.7 to 30.3)
SatDNA cPCR vs kDNA cPCR	Total	−18.6 (−29.9 to −6.0)*	7.3 (−0.6 to 17.3)
	Infants	−5.9 (−22.9 to 10.9)	13.3 (−0.6 to 29.7)
	Adults	−26.9 (−43.8 to −7.6)*	0.0 (−13.3 to 13.3)
kDNA qPCR vs kDNA cPCR	Total	9.3 (−0.5 to 18.8)	−12.7 (−23.7 to −3.2)*
	Infants	0.0 (−14.3 to 14.3)	−10.0 (−24.4 to 3.3)
	Adults	15.4 (−0.2 to 29.4)	−16.0 (−34.7 to 0.4)
SatDNA qPCR vs SatDNA cPCR	Total	20.9 (7.7 to 32.6)*	−3.6 (−12.3 to 3.4)
	Infants	5.9 (−10.9 to 22.9)	−3.3 (−16.7 to 8.3)
	Adults	30.8 (10.6 to 47.7)*	−4 (−19.5 to 9.7)

*The differences in sensitivity and specificity and the 95% CIs between PCR methods were calculated using a three-way approach in StatisPro software version 3.02.2 (Analyse-it Software Ltd. and CLSI, Wayne, PA). If the 95% CI does not include 0, then there are significant differences between methods.

cPCR, end-point PCR; kDNA, kinetoplastid DNA; qPCR, real-time quantitative PCR; SatDNA, satellite DNA.

and within-laboratory precision (St) in terms of SD compared with the published claims.[22,23] If the estimated SD was less than or equal to the verification value, data were consistent with the reported claim, and the claim was verified. Furthermore, trueness was estimated in terms of bias between measured parasitic loads and the established reference value (cell count obtained in a Z2 Coulter Particle Count and Size Analyzer, in triplicate and using different parasite dilutions). If the calculated verification interval for bias included the assigned value, then trueness was demonstrated.

Results

Comparative Study of PCR Methods

A total of 98 GEB samples were analyzed: 43 obtained from patients with confirmed CD reactive serologic tests and 55 from individuals with nonreactive serologic tests for *T. cruzi* as negative controls to address specificity. The results of the comparative analysis are summarized in Table 2.

In all cases, the sensitivity for *T. cruzi* DNA detection was higher in samples obtained from infants than in those from adult patients. Overall, qPCR procedures had better diagnostic sensitivity than cPCR ones when comparing each molecular target (SatDNA: 60%; 95% CI, 46%−74%; versus 40%; 95% CI, 26%−54%; kDNA: 67%; 95% CI, 53%−80%; versus 58%; 95% CI, 43%−72%), with a slight decrease in their diagnostic specificity (SatDNA: 96%; 95% CI, 88%−99%; versus 100%; 95% CI, 93%−100%; kDNA: 80%; 95% CI, 68%−88%; versus 93%; 95% CI, 83%−97%).

In general, the diagnostic efficiency (the proportion of true results) was higher in samples obtained from infants than in those from adult patients (Table 2). In particular, for qPCR methods, the efficiency was higher in the SatDNA than in the kDNA qPCR, especially when analyzing samples obtained from infants. On the other hand, cPCR efficiency was higher when SatDNA was amplified from samples from infants and when kDNA was detected in samples from adults.

The significant differences in sensitivity and specificity obtained by the three-way comparison approach are given in Table 3. No significant differences in clinical sensitivity were found between SatDNA and kDNA targets when amplified by qPCR from samples from infants or adults. In contrast, the kDNA cPCR was significantly more sensitive

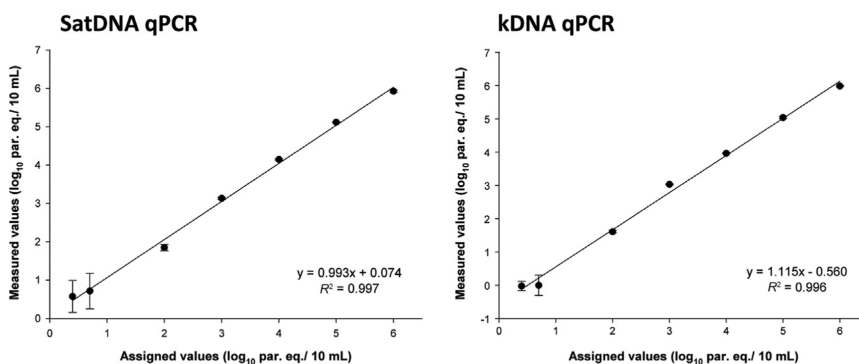

Figure 1 Analytical verification of the anticipated reportable range of the satellite DNA (SatDNA) and kinetoplastid DNA (kDNA) real-time quantitative PCR (qPCR) methods. The assays were performed with spiked guanidine-EDTA-blood samples that contained 10^5 to 0.25 parasite equivalents per milliliter of blood (par. eq./mL), tested in triplicate. Assigned values were plotted on the *x* axis versus measured values (converted to $\log_{10}$) on the *y* axis using SigmaPlot version 10.0 (SPSS, Chicago, IL).

Table 4 Verification of the Precision and Trueness of the Modified qPCR Assays

Method	Estimate*	0.25 par. eq./mL			
		SD Claim	Verification Limit	SD	CV, %
SatDNA qPCR	Repeatability	ND	ND	ND	ND
	Within laboratory	ND	ND	ND	ND
	Bias, mean (interval), $\log_{10}$ par. eq./10 mL	ND			
kDNA qPCR	Repeatability	0.278	0.410	0.372†	−172.2
	Within laboratory	0.273	0.409	0.396*†	−183.6
	Bias, mean (interval), $\log_{10}$ par. eq./10 mL)	−0.232 (−0.780 to 1.560)			

(*table continues*)

*CLSI EP15-A2 guideline[30] and StatisPro software version 3.02.2 (Analyse-it Software Ltd. and CLSI, Wayne, PA) were used to calculate the repeatability and within-laboratory SDs and the bias of kDNA and SatDNA qPCR assays compared with the reported claims.[22,23]

†If the SD is less than or equal to the limit of verification value, the claim is verified.

kDNA, kinetoplastid DNA; ND, not done; par. eq./mL, parasite equivalents per milliliter of blood; qPCR, real-time quantitative PCR; SatDNA, satellite DNA.

to detect *T. cruzi* DNA in samples from adults than the SatDNA cPCR. Overall, a greater number of false-positive results, and consequently lower specificity and positive predictive values, were obtained in kDNA-based PCRs (significant differences were found between the SatDNA and the kDNA qPCRs and between the cPCR and qPCR kDNA methods), particularly when samples from infants were processed (Tables 2 and 3).

Analytical Verification of the Performance of qPCR Assays

Anticipated Reportable Range

A linearity experiment was performed with a panel of eight spiked GEB dilutions that spanned 10^5 to 0.25 par. eq./mL of blood. Linear regression analysis gave the equations $y = 0.993x + 0.074$ ($R^2 = 0.997$) for the SatDNA qPCR and $y = 1.115x - 0.560$ ($R^2 = 0.996$) for the kDNA qPCR. Accordingly, the anticipated reportable range was verified between 0.25 and 10^5 par. eq./mL for both qPCR methods (Figure 1).

LOD

Spiked GEB samples that contained 0.25, 0.7, and 1 par. eq./mL of blood were prepared to perform a replicate test to verify the reported LODs for both qPCR assays. Replicates that contained 0.7 par. eq./mL (reported $LOD_{95\%}$ concentration for the SatDNA qPCR) and 1 par. eq./mL gave 87.5% ($n = 21/24$) and 100% ($n = 24/24$) positive results in the SatDNA qPCR, respectively. Meanwhile, replicates with 0.25 par. eq./mL (reported $LOD_{95\%}$ concentration for the kDNA qPCR) and 0.7 par. eq./mL gave 83.3% ($n = 20/24$) and 100% ($n = 24/24$) positive results in the kDNA qPCR, respectively. Consequently, the LOD of the modified SatDNA and kDNA qPCR assays was estimated using the PODLOD.xls application. The qPCR results obtained for the 15 replicates of the 0.5−, 10−, and 1000−par. eq./mL GEB samples from the precision analysis and 24 additional replicates that contained 0.125 par. eq./mL (only for the kDNA qPCR) were used together with the LOD verification results described above to estimate the LOD values of the modified qPCRs. The calculated $LOD_{95\%}$ was 0.87 par. eq./mL (95% CI, 0.62−1.24 par. eq./mL) for the SatDNA qPCR and 0.43 par. eq./mL (95% CI, 0.32−0.59 par. eq./mL) for the kDNA qPCR.

Precision and Trueness

Three replicates of each concentration of spiked GEB samples (0.5, 10, and 1000 par. eq./mL for the SatDNA qPCR and 0.25, 10, and 1000 par. eq./mL for the kDNA qPCR) were tested for 5 consecutive days (15 replicates in total for each concentration). Sr and St were calculated as recommended by the CLSI EP15-A2 guideline and compared with the reported claims (Table 4). The calculated Sr and St were lower than their verification values in both modified qPCR methods when the highest and lowest concentrations tested were analyzed, and thus the reported claims for these concentrations were verified. In contrast, for 10 par. eq./mL, St was not verified for either method, and Sr was verified only for the SatDNA qPCR, although the difference with the reported SD claim and the CV did not exceed 1 $\log_{10}$ or 20%, respectively (Table 4).

The qPCR results obtained for the precision experiments were also used to assess trueness, which was estimated in terms of bias in parasitic loads measured in $\log_{10}$ par. eq./10 mL of blood relative to an accepted reference value. The calculated interval for bias included the assigned values (0.39, 0.69, 2, and 4 $\log_{10}$ par. eq./10 mL for 0.25, 0.5, 10, and 1000 par. eq./mL, respectively) for all the samples tested, demonstrating trueness in both qPCR methods (Table 4).

Discussion

Congenital infection is the third main route of *T. cruzi* transmission and the first cause of new cases of CD in Argentina and in nonendemic areas worldwide. Actually, 1078 and 1457 annual new cases of CD have been estimated to occur in this country because of vectorial and congenital

Table 4 (*continued*)

0.5 par. eq./mL				10 par. eq./mL				1000 par. eq./mL			
SD Claim	Verification Limit	SD	CV, %	SD Claim	Verification Limit	SD	CV, %	SD Claim	Verification Limit	SD	CV, %
0.616	0.990	0.557†	57.3	0.177	0.261	0.228†	10.0	0.086	0.127	0.026†	0.6
0.551	0.842	0.557†	57.3	0.153	0.200	0.206	9.0	0.078	0.109	0.026†	0.6
0.905 (−0.505 to 1.885)				2.282 (0.856 to 3.144)				4.193 (2.869 to 5.131)			
ND	ND	ND	ND	0.156	0.230	0.372	19.2	0.081	0.119	0.051*†	1.3
ND	ND	ND	ND	0.217	0.270	0.367	19.0	0.132	0.170	0.054*†	1.4
ND				1.873 (0.838 to 3.162)				4.002 (2.868 to 5.132)			

transmission, respectively.[1] Treatment of children who acquired CD congenitally is always effective when pursued in infants within the first years of life. Therefore, early diagnosis of the infection may play an essential role in controlling the disease burden in this population. In addition to the standard tests, a PCR performed in blood samples from neonates of infected mothers might improve such diagnosis.[5] In this way, the Instituto Nacional de Parasitología Dr. Mario Fatala Chaben has initiated a technology transfer process of PCR tests for the diagnosis of congenital CD to be implemented in the Public Health System (C.I.C., C.L.-A., J.C.R., K.S., and S.S.-E., unpublished data).

To select the most suitable PCR methods to be implemented during the transfer process, we compared the performance of the four PCR assays most widely used for CD diagnosis: two end-point PCRs and two duplex qPCRs that target *T. cruzi* SatDNA and kDNA. The end-point PCR methods have demonstrated a predictive role in the diagnosis of congenital CD,[21] whereas qPCR assays have been widely applied and proved to be useful to detect treatment failure in clinical trials with antiparasitic drugs and to monitor reactivation of infection in immunocompromised patients because of organ transplantation or HIV coinfection.[33–37]

The sensitivity of *T. cruzi* DNA detection was higher in samples obtained from infants than in those from adult patients, independently of the type or the target of the method analyzed (Table 2). This finding reflects the characteristic higher parasitic loads found in samples from recent congenital transmission and acute CD cases compared with patients with chronic CD.[22–24] All infant samples analyzed in this study were from blood samples collected after the infants were 10 month of age, when bloodstream parasite loads in congenitally infected children are significantly lower than those measured during the first months of newborn life.[20] This fact explains the relative low diagnostic sensitivity found in this group compared with previous reports.[17,18,21] The decision to use these samples was based on the difficulties and limitations involved in assembling a sufficient number of earlier follow-up samples from infants, especially positive ones, in our institute at the time this study was conducted.

Clinical sensitivity was higher when kDNA rather than SatDNA was amplified by qPCR or cPCR assays (Table 2). This is in agreement with previous reports of higher analytical and clinical sensitivities in kDNA- than in SatDNA-based PCR methods for all *T. cruzi* discrete typing units.[12,23] In contrast, a greater number of false-positive results, and consequently a lower specificity, were obtained in kDNA-based PCRs (Tables 2 and 3). In addition, kDNA-based PCR methods have revealed false-positive results attributable to the presence of *Trypanosoma rangeli*; consequently, their use is discouraged for diagnosing *T. cruzi* infection in some Central and South American countries, which are endemic for this related trypanosomatid.[23]

Overall, qPCR methods had a better performance than cPCR methods, particularly in terms of sensitivity. Our findings, together with recommendations made by a consensus of experts in molecular diagnosis of *T. cruzi*, suggest that qPCR methods would be the most appropriate to be implemented during the transfer process. qPCR methods encompass technical advantages, such as i) closed-tube or closed-well single-round PCR reaction, which minimizes carryover contamination; ii) automation and possibility of scaling up; iii) shorter time in processing samples and obtaining results; iv) simultaneous detection of target DNA and internal amplification controls in TaqMan probe−based methods; and v) ability to obtain quantitative data.

Once selected, slight modifications were introduced into the original kDNA and SatDNA qPCR procedures previously described[22,23] to improve the performance of these methods on the basis of their operative advantages in a transfer process. UNG was added to the reaction mix as a carryover contamination control to decrease the risk of false-positive results. The *RNase P* gene was also used as internal amplification control in both qPCR methods because of the availability of a standardized commercial kit and the fact that, in this instance, these qPCRs are not intended to be used for quantification purposes, in which case the use of a heterologous extrinsic control would be the most appropriate.[22] To provide empirical evidence that such

modifications and the current laboratory conditions did not significantly vary the analytical performance of these qPCRs, both methods were subjected to an analytical verification protocol based on international guidelines compared with the reported specifications.[22,23] Analytical procedures to verify the anticipated reportable range, LOD, and precision were applied. In addition, the EP15-A2 guideline was used to assess trueness.[31]

The anticipated reportable range was found between 0.25 and 10^5 par. eq./mL for both qPCR assays (Figure 1), verifying the published claims. The reported LOD concentrations for the original methods (0.7 par. eq./mL for the SatDNA qPCR and 0.25 par. eq./mL for the kDNA qPCR) could not verify the $LOD_{95\%}$ for the modified methods. Instead, they verified the $LOD_{87\%}$ and $LOD_{83\%}$, respectively. The LODPOD.xls application was used to calculate the LOD of the modified qPCRs, which was estimated to be 0.87 and 0.43 par. eq./mL of blood for the SatDNA and kDNA qPCRs, respectively. The differences found in the LOD values with respect to those reported for the original methods (<0.2 par. eq./mL) could be attributable to the slight modifications introduced but also to the intrinsic error of the cell counting method, high imprecision of qPCR methods at low target concentrations,[25] different approaches used for the estimation of $LOD_{95\%}$,[26,38] or even only to the different laboratory conditions.

Precision (estimated for Sr and St) was verified for the highest and the lowest concentrations tested in both modified qPCR methods, although the lowest dilutions (0.5 and 0.25 par. eq./mL for the SatDNA and the kDNA qPCRs, respectively) exhibited very high CV values, as expected for samples that are indeed below the LOD of these methods (Table 4). We verified the precision at these low parasite concentrations because they were the lowest tested during the validation process of the original methods.[22,23] On the other hand, precision could not be verified for 10 par. eq./mL dilution, except for Sr in the SatDNA qPCR, although the differences with the reported SD claim and CV did not exceed 1 $\log_{10}$ or 20%, respectively (Table 4). Although it is not mandatory to verify the precision claim in qualitative assays, which is the way these qPCRs will be implemented in the Public Health System, the 10−par. eq./mL concentration will be tested during the next 6 months to fully characterize the modified methods. Trueness, which was estimated in terms of bias, was verified at all the concentrations tested by both qPCR assays.

Although minor differences were found in the verification of analytical parameters for the modified qPCRs compared with those reported for the original assays, they would not affect the qualitative diagnostic properties of the modified qPCR methods in any way. These differences could be attributable to the slight modifications introduced (UNG incorporation and Rnase P as internal amplification control) and to the differences in laboratory conditions and facilities, such as the qPCR device, the operators, and the starting parasite count. Such variability is indeed expected to occur during the transfer process to the different laboratories of the National Public Health network. The analytical verification process conducted in this work provides critical knowledge of the technology transfer process planned to cover regional laboratories with known installed facilities, which must apply their own verification protocols on the transfer method.

In summary, the results of this work allowed for selection of the two most appropriate PCR methods to be implemented during the transfer process of the four initially compared. Both qPCR methods had a good performance in the detection of *T. cruzi* DNA in blood samples, with slight differences in terms of sensitivity and specificity, which led us to consider a tentative molecular diagnostic algorithm that includes a screening (more sensitive) and a confirmatory (more specific) technique. On the basis of that and the potential advantages of having two different molecular targets to confirm the diagnosis, as recommended by a consensus of experts in molecular diagnosis of *T. cruzi*, we decided to analytically verify and clinically validate both qPCR methods. Currently, our laboratory is performing an implementation study to evaluate both SatDNA and kDNA qPCRs in a new algorithm that combines parasitologic and serologic methods with molecular assays for the diagnosis of congenital CD (C.I.C., C.L.-A., J.C.R., K.S., and S.S.-E., unpublished data). That work will reveal whether one or both qPCR methods will be needed to confirm the molecular diagnosis of congenital infection and will allow us to make a final recommendation to the national network and national guidelines of diagnosis.

Early diagnosis of congenital CD is of utmost importance to guarantee a curative treatment of infant patients. The availability of simpler and more accurate diagnostic methods easily implemented in primary health care settings could provide tools for the timely treatment of infected newborns.

Acknowledgments

We thank Drs. Jacqueline Bua, Gabriela García, Cristina Maidana, and Alina Perrone from Instituto Nacional de Parasitología Dr. Mario Fatala Chaben and María de los Angeles Curto from Institute of Investigation in Genetic Engineering and Molecular Biology “Dr. Héctor N. Torres” for providing and counting *T. cruzi* stocks.

References

1. World Health Organization: Chagas disease in Latin America: an epidemiological update based on 2010 estimates. Wkly Epidemiol Rec 2015, 90:33−43
2. World Health Organization: Investing to overcome the global impact of neglected tropical diseases: Third WHO report on neglected tropical diseases. Atlanta, GA, World Health Organization, 2015
3. Buekens P, Almendares O, Carlier Y, Dumonteil E, Eberhard M, Gamboa-Leon R, James M, Padilla N, Wesson D, Xiong X: Mother-to-child transmission of Chagas’ disease in North America: why don’t we do more? Matern Child Health J 2008, 12:283−286

4. Howard EJ, Xiong X, Carlier Y, Sosa-Estani S, Buekens P: Frequency of the congenital transmission of Trypanosoma cruzi: a systematic review and meta-analysis. BJOG 2014, 121:22−33
5. Carlier Y, Sosa-Estani S, Luquetti AO, Buekens P: Congenital Chagas disease: an update. Mem Inst Oswaldo Cruz 2015, 110:363−368
6. Sosa-Estani S, Dri L, Touris C, Abalde S, Dell'arciprete A, Braunstein J: Vectorial and congenital transmission of Trypanosoma cruzi in Las Lomitas, Formosa. Medicina (B Aires) 2009, 69:424−430
7. Chuit R, Segura EL: Results of control of Chagas' disease in Argentina. Rev Fed Arg Cardiol 2012, 41:151−155
8. Gomes YM, Lorena VM, Luquetti AO: Diagnosis of Chagas disease: what has been achieved? What remains to be done with regard to diagnosis and follow up studies? Mem Inst Oswaldo Cruz 2009, 104 Suppl 1:115−121
9. Ministerio de Salud de la Nación: Guías para la atención al paciente infectado con Trypanosoma cruzi (Enfermedad de Chagas). Buenos Aires, Argentina, 2012. Article in Spanish. Available at http://www.msal.gob.ar/chagas/images/stories/Equipos/Guia_Nacional_Chagas_version_27092012.pdf
10. WHO Expert Committee: Control of Chagas disease. World Health Organ Tech Rep Ser 2002, 905:i−vi, 1−109
11. Ministerio de Salud de la Nación Argentina: Normas para el Diagnóstico de la Infección Chagásica. Buenos Aires, 1999. Min. Resol. No. 523/97, ed 2
12. Schijman AG, Bisio M, Orellana L, Sued M, Duffy T, Mejia Jaramillo AM, et al: International study to evaluate PCR methods for detection of Trypanosoma cruzi DNA in blood samples from Chagas disease patients. PLoS Negl Trop Dis 2011, 5:e931
13. Carlier Y, Torrico F, Sosa-Estani S, Russomando G, Luquetti A, Freilij H, Albajar Vinas P: Congenital Chagas disease: recommendations for diagnosis, treatment and control of newborns, siblings and pregnant women. PloS Negl Trop Dis 2011, 5:e1250
14. Sosa-Estani S: Congenital transmission of Trypanosoma cruzi infection in Argentina. Rev Soc Bras Med Trop 2005, 38 Suppl 2:29−32
15. De Rissio AM, Riarte AR, Garcia MM, Esteva MI, Quaglino M, Ruiz AM: Congenital Trypanosoma cruzi infection. Efficacy of its monitoring in an urban reference health center in a non-endemic area of Argentina. Am J Trop Med Hyg 2010, 82:838−845
16. Russomando G, de Tomassone MM, de Guillen I, Acosta N, Vera N, Almiron M, Candia N, Calcena MF, Figueredo A: Treatment of congenital Chagas disease diagnosed and followed-up by the polymerase chain reaction. Am J Trop Med Hyg 1998, 59:487−491
17. Schijman AG, Altcheh J, Burgos JM, Biancardi M, Bisio M, Levin MJ, Freilij H: A etiological treatment of congenital Chagas' disease diagnosed and monitored by the polymerase chain reaction. J Antimicrob Chemother 2003, 52:441−449
18. Virreira M, Torrico F, Truyens C, Alonso-Vega C, Solano M, Carlier Y, Svoboda M: Comparison of polymerase chain reaction methods for reliable and easy detection of congenital Trypanosoma cruzi infection. Am J Trop Med Hyg 2003, 68:574−582
19. Burgos JM, Altcheh J, Petrucelli N, Bisio M, Levin MJ, Freilij H, Schijman AG: Molecular diagnosis and treatment monitoring of congenital transmission of Trypanosoma cruzi to twins of a triplet delivery. Diagn Microbiol Infect Dis 2009, 65:58−61
20. Bua J, Volta BJ, Perrone AE, Scollo K, Velázquez EB, Ruiz AM, De Rissio AM, Cardoni RL: How to improve the early diagnosis of Trypanosoma cruzi infection: relationship between validated conventional diagnosis and quantitative DNA amplification in congenitally infected children. PloS Negl Trop Dis 2013, 7:e2476
21. Velázquez EB, Rivero R, De Rissio AM, Malagrino N, Esteva MI, Riarte AR, Ruiz AM: Predictive role of polymerase chain reaction in the early diagnosis of congenital Trypanosoma cruzi infection. Acta Trop 2014, 137:195−200
22. Duffy T, Cura CI, Ramirez JC, Abate T, Cayo NM, Parrado R, Diaz Bello Z, Velazquez E, Muñoz-Calderon A, Juiz NA, Basile J, Garcia L, Riarte A, Nasser JR, Ocampo SB, Yadon ZE, Torrico F, Alarcón de Noya B, Ribeiro I, Schijman AG: Analytical performance of a Multiplex Real-Time PCR assay using TaqMan probes for quantification of Trypanosoma cruzi satellite DNA in blood samples. PloS Negl Trop Dis 2013, 7:e2000
23. Ramírez JC, Cura CI, da Cruz Moreira O, Lages-Silva E, Juiz N, Velázquez E, et al: Analytical validation of quantitative real-time PCR methods for quantification of Trypanosoma cruzi DNA in blood samples from Chagas disease patients. J Mol Diagn 2015, 17:605−615
24. Duffy T, Bisio M, Altcheh J, Burgos JM, Diez M, Levin MJ, Favaloro RR, Freilij H, Schijman AG: Accurate real-time PCR strategy for monitoring bloodstream parasitic loads in Chagas disease patients. PloS Negl Trop Dis 2009, 3:e419
25. Burd EM: Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev 2010, 23:550−576
26. Rodriguez M, Irazu L, Gomes K, Stupka J: Development and validation of in house or modified molecular methods. Implementation experience in the public health field. Acta Bioquím Clín Latinoam 2012, 46:351−608
27. Clinical and Laboratory Standards Institute (CLSI): User protocol for evaluation of qualitative test performance. Approved guideline, ed 2. EP12-A2. Wayne, PA, 2008
28. Clinical and Laboratory Standards Institute (CLSI): Evaluation of the linearity of quantitative measurement procedures: a statistical approach. Approved guideline EP6-A. Wayne, Pennsylvania, 2003
29. International Organization for Standardization: Microbiology of the food chain—method validation—part 3: protocol for the verification of reference and alternative methods implemented in a single laboratory. Geneva, Switzerland: International Organization for Standardization, 2014, ISO/WD 16140-3
30. Wilrich C, Wilrich PT: Estimation of the POD function and the LOD of a qualitative microbiological measurement method. J AOAC Int 2009, 92:1763−1772
31. Clinical and Laboratory Standards Institute (CLSI): User verification of performance for precision and trueness. Approved guideline, ed 2. EP15-A2. Wayne, Pennsylvania, 2003
32. Clinical and Laboratory Standards Institute (CLSI): Specifications for immunological testing for infectious diseases. Approved guideline, ed 2. I/LA18-A2. Wayne, Pennsylvania, 2001
33. de Freitas VL, da Silva SC, Sartori AM, Bezerra RC, Westphalen EV, Molina TD, Teixeira AR, Ibrahim KY, Shikanai-Yasuda MA: Real-time PCR in HIV/Trypanosoma cruzi coinfection with and without Chagas disease reactivation: association with HIV viral load and CD4 level. PLoS Negl Trop Dis 2011, 5:e1277
34. Qvarnstrom Y, Schijman AG, Veron V, Aznar C, Steurer F, da Silva AJ: Sensitive and specific detection of Trypanosoma cruzi DNA in clinical specimens using a multi-target real-time PCR approach. PloS Negl Trop Dis 2012, 6:e1689
35. Molina I, Gómez i Prat J, Salvador F, Treviño B, Sulleiro E, Serre N, Pou D, Roure S, Cabezos J, Valerio L, Blanco-Grau A, Sánchez-Montalvá A, Vidal X, Pahissa A: Randomized trial of posaconazole and benznidazole for chronic Chagas' disease. N Engl J Med 2014, 370:1899−1908
36. Pinazo MJ, Thomas MC, Bua J, Perrone A, Schijman AG, Viotti RJ, Ramsey JM, Ribeiro I, Sosa-Estani S, López MC, Gascon J: Biological markers for evaluating therapeutic efficacy in Chagas disease, a systematic review. Expert Rev Anti Infect Ther 2014, 12:479−496
37. Álvarez MG, Hernández Y, Bertocchi G, Fernández M, Lococo B, Ramírez JC, Cura C, Albizu CL, Schijman A, Abril M, Sosa-Estani S, Viotti R: New scheme of intermittent benznidazole administration in patients chronically infected with Trypanosoma cruzi: a pilot short-term follow-up study with adult patients. Antimicrob Agents Chemother 2015, 60:833−837
38. Rodriguez M, Irazu L, Bueno N, Bonaventura R, Mariotti A, Freire M.C. [The estimation of the limit of detection in molecular methods of virological diagnosis. Differences in the application of the Probit and POD/LOD models]. Spanish. XI Argentine Congress of Virology, II Latin American Congress of Virology, IV Symposium on Clinical Virology, II Symposium on Veterinary Virology "Dr. José L. La Torre" [abstract], Buenos Aires, Argentina, 2015.