The Journal of Molecular Diagnostics, Vol. 17, No. 5, September 2015

the Journal of Molecular Diagnostics

jmd.amjpathol.org

Analytical Validation of Quantitative Real-Time PCR Methods for Quantification of *Trypanosoma cruzi* DNA in Blood Samples from Chagas Disease Patients

Juan Carlos Ramírez,[*] Carolina Inés Cura,[*] Otacilio da Cruz Moreira,[†] Eliane Lages-Silva,[‡] Natalia Juiz,[*] Elsa Velázquez,[§] Juan David Ramírez,[¶] Anahí Alberti,[‖] Paula Pavia,[**] María Delmans Flores-Chávez,[††] Arturo Muñoz-Calderón,[‡‡] Deyanira Pérez-Morales,[§§] José Santalla,[¶¶] Paulo Marcos da Matta Guedes,[‖‖] Julie Peneau,[***] Paula Marcet,[†††] Carlos Padilla,[‡‡‡] David Cruz-Robles,[§§§] Edward Valencia,[¶¶¶] Gladys Elena Crisante,[‖‖‖] Gonzalo Greif,[****] Inés Zulantay,[††††] Jaime Alfredo Costales,[‡‡‡‡] Miriam Alvarez-Martínez,[§§§§] Norma Edith Martínez,[¶¶¶¶] Rodrigo Villarroel,[‖‖‖‖] Sandro Villarroel,[*****] Zunilda Sánchez,[†††††] Margarita Bisio,[*] Rudy Parrado,[*****] Lúcia Maria da Cunha Galvão,[‖‖] Antonia Cláudia Jácome da Câmara,[‖‖] Bertha Espinoza,[§§] Belkisyole Alarcón de Noya,[‡‡] Concepción Puerta,[**] Adelina Riarte,[§] Patricio Diosque,[‖] Sergio Sosa-Estani,[§] Felipe Guhl,[¶] Isabela Ribeiro,[‡‡‡‡‡] Christine Aznar,[***] Constança Britto,[†] Zaida Estela Yadón,[§§§§§] and Alejandro G. Schijman[*]

From the Laboratory of Molecular Biology of Chagas Disease (LaBMECh),[*] Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET), Buenos Aires, Argentina; the Laboratory of Molecular Biology of Endemic Diseases,[†] Instituto Oswaldo Cruz/Fiocruz, Rio de Janeiro, Brazil; the Laboratory of Discipline of Parasitology,[‡] Universidade Federal do Triângulo Mineiro, Uberaba, Brazil; the National Institute of Parasitology "Dr. Mario Fatala Chabén",[§] Buenos Aires, Argentina; the Center for Research in Tropical Microbiology and Parasitology,[¶] Universidad de los Andes, Bogota, Colombia; the Institute of Experimental Pathology,[‖] CONICET-Universidad Nacional de Salta, Salta, Argentina; the Laboratory of Molecular Parasitology,[**] Pontificia Universidad Javeriana, Bogota, Colombia; the National Center for Microbiology,[††] Instituto de Salud Carlos III, Madrid, Spain; the Institute of Tropical Medicine,[‡‡] Universidad Central de Venezuela, Caracas, Venezuela; the Biomedical Research Institute,[§§] Universidad Nacional Autónoma de México, Mexico DF, Mexico; the Laboratory of Parasitology and Molecular Biology,[¶¶] Instituto Nacional de Laboratorios en Salud, La Paz, Bolivia; the Department of Microbiology and Parasitology,[‖‖] Universidade Federal do Rio Grande do Norte, Natal, Brazil; the Hospital and University Laboratory-CH Andrée Rosemon,[***] Cayenne, French Guiana; the Division of Parasitic Diseases and Malaria,[†††] Centers for Disease Control and Prevention, Atlanta, Georgia; the National Center for Public Health,[‡‡‡] Instituto Nacional de Salud, Lima, Peru; the Laboratory of Genomics,[§§§] Instituto Nacional de Cardiología "Ignacio Chávez", Mexico DF, Mexico; the Laboratory for Research in Infectious Diseases,[¶¶¶] Universidad Peruana Cayetano Heredia, Lima, Peru; the Department of Biology,[‖‖‖] Universidad de los Andes, Merida, Venezuela; the Molecular Biology Unit,[****] Instituto Pasteur de Montevideo, Montevideo, Uruguay; the Basic Clinical Parasitology Laboratory,[††††] Universidad de Chile, Santiago, Chile; the Research Center for Infectious Diseases,[‡‡‡‡] Pontificia Universidad Católica de Ecuador, Quito, Ecuador; the Microbiology Department,[§§§§] Hospital Clinic and Barcelona Centre for International Health Research (CRESIB), Barcelona, Spain; the State Laboratory of Public Health,[¶¶¶¶] Acapulco, Mexico; the Biomedical Department ,[‖‖‖‖] Instituto de Salud Pública, Santiago, Chile; the Laboratory of Molecular Biology,[*****] Universidad Mayor de San Simón, Cochabamba, Bolivia; the Research Institute for Health Sciences,[†††††] Universidad Nacional de Asunción, Asuncion, Paraguay; the Drugs for Neglected Diseases Initiative (DNDi),[‡‡‡‡‡] Geneva, Switzerland; and the Communicable Diseases and Health Analysis Department,[§§§§§] Pan American Health Organization/World Health Organization (PAHO/WHO), Rio de Janeiro, Brazil

Accepted for publication April 3, 2015.

Address correspondence to Alejandro G. Schijman, M.D., Vuelta de Obligado 2490, C1428ADN, Buenos Aires, Argentina. E-mail: schijman@dna.uba.ar.

An international study was performed by 26 experienced PCR laboratories from 14 countries to assess the performance of duplex quantitative real-time PCR (qPCR) strategies on the basis of TaqMan probes for detection and quantification of parasitic loads in peripheral blood samples from Chagas disease patients. Two methods were studied: Satellite DNA (SatDNA) qPCR and kinetoplastid DNA (kDNA) qPCR. Both methods included an internal amplification control. Reportable range, analytical sensitivity, limits of detection and quantification, and precision were estimated according to international guidelines. In addition, inclusivity and exclusivity were estimated with DNA from stocks representing the different *Trypanosoma cruzi* discrete typing units and *Trypanosoma rangeli* and *Leishmania* spp. Both methods were challenged against 156 blood samples provided by the participant laboratories, including samples from acute and chronic patients with varied clinical findings, infected by oral route or vectorial transmission. kDNA qPCR showed better analytical sensitivity than SatDNA qPCR with limits of detection

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http://dx.doi.org/10.1016/j.jmoldx.2015.04.010

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of 0.23 and 0.70 parasite equivalents/mL, respectively. Analyses of clinical samples revealed a high concordance in terms of sensitivity and parasitic loads determined by both SatDNA and kDNA qPCRs. This effort is a major step toward international validation of qPCR methods for the quantification of *T. cruzi* DNA in human blood samples, aiming to provide an accurate surrogate biomarker for diagnosis and treatment monitoring for patients with Chagas disease. *(J Mol Diagn 2015, 17: 605–615; http://dx.doi.org/10.1016/j.jmoldx.2015.04.010)*

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi,* affects mostly the poor populations in 21 countries of the Americas, where close to 7 to 8 million people are infected, 25 million are at risk, and 10 thousand deaths are recorded annually (World Health Organization, *www.who.int/mediacentre/factsheets/fs340/en*, last accessed November 1, 2014).[1] In recent years, this neglected tropical disease is becoming a global concern because of the increasing migration from Latin America to nonendemic countries from Europe and North America.[2]

Complex interactions between the genetic background of the parasite and the host and environmental and epidemiologic factors determine the outcome of the infection. In the acute phase of CD the symptoms are variable, and in most cases resolve spontaneously after some weeks. Appropriate treatment can eliminate the parasite during this phase, but the infection is only recognized in 1% to 2% of infected persons during the acute phase. In the chronic phase, approximately 70% of seropositive persons are asymptomatic, whereas 30% ultimately develop serious cardiac and/or digestive disorders several years or decades later, and necrotizing inflammatory injuries in the central nervous system in cases of CD reactivation under immunodepression. Each year, 2% to 3% of symptomatic persons start to present manifestations that can rapidly evolve to sudden death. However, the factors that govern the progression of chronic CD remain unknown, and no prognostic markers are available.[3]

Accurate diagnostics tools and surrogate markers of parasitologic response to treatment are priorities in CD research and development.[4] To develop an accurate laboratory tool for diagnosis and treatment follow-up, several difficulties need to be addressed, such as the low and intermittent number of circulating parasites during the chronic phase of infection and parasite genotype diversity, because six discrete typing units (DTUs), TcI to TcVI, are unevenly distributed in different endemic regions.[5]

Supported by CDR/Small Grant Programme PAHO/WHO (A.G.S.) and partially by CONICET grant PIP 112-200801-02915 and the National Agency of Science and Technology grant PICT 33955.

The findings and conclusions of this manuscript are those of the authors and do not necessarily represent the views of the US Centers for Disease Control and Prevention (Atlanta, GA).

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

Disclosures: A.G.S. is a research member of CONICET. Z.E.Y. is Regional Advisor on Communicable Diseases Research and Health Analysis, Pan American Health Organization. B.A.d.N., A.R., S.S.-E., F.G., I.R., and A.G.S. are members of NHEPACHA Network. Life Technologies Argentina provided access to an ABI StepOne Real-Time PCR machine.

Quantitative real-time PCR (qPCR)-based assays may fill these gaps, but their application in the clinical practice requires prior analytical and clinical validation studies.[6,7] So far, a few real-time PCR strategies have been developed for *T. cruzi* DNA detection and quantification in CD patients.[8–11]

As part of the Small Grants Programme (joined initiative of Communicable Diseases Research/Pan-American Health Organization) and The Special Programme for Research and Training in Tropical Diseases/United Nations Development Program/United Nations Children's Fund/World Bank/World Health Organization, an international study was performed by 26 experienced PCR laboratories from 14 countries to assess the performance of duplex qPCR strategies on the basis of TaqMan probes for detection and quantification of the parasite loads in blood samples of CD patients.

Materials and Methods

Ethics Statement

The studies in which the samples were collected were approved by the ethical committees of the participating institutions, according to the principles expressed in the Declaration of Helsinki. Written informed consent forms were signed by the adult study subjects and from parents/guardians on behalf of all minor subjects. All samples were pre-existent at the time of this international study and were anonymized before being processed.

Spiked Blood Samples

Seronegative human blood samples were spiked with cultured epimastigotes of Sylvio X10 and CL-Brener stocks (TcId and TcVI, respectively) and were immediately mixed with one volume of guanidine hydrochloride 6 mol/L EDTA 0.2 mol/L buffer, pH 8.00 (GE).

Patients and Blood Specimens

Peripheral blood samples from 156 CD patients were distributed into eight groups according to their geographic origin, as follows. Group 1 (G1) included samples from four seropositive patients from Mexico, two patients with acute CD (G1a) and two patients with asymptomatic chronic CD (G1b). Group 2 (G2) included samples from two patients from French Guiana with acute CD acquired by oral

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transmission. One patient was positive and the other patient was negative for IgG serologic studies. Both patients experienced cardiac symptoms and were infected with TcI. Group 3 (G3) included samples from five seropositive patients from Bolivia; two patients with acute CD acquired by oral transmission (G3a) and three patients with asymptomatic chronic CD acquired from vectors or congenitally (G3b). Group 4 (G4) included samples from five seropositive patients from Venezuela with acute CD acquired by oral transmission. Two of these patients were infected with TcI. Group 5 (G5) included samples from 13 seropositive patients from Colombia with asymptomatic chronic CD acquired from vectors or congenitally. Five of these patients were infected with TcI. Group 6 (G6) included samples from 21 Bolivian seropositive patients, resident in Spain, with chronic CD acquired from vectors or congenitally. One patient experienced digestive symptoms and the others were asymptomatic. Group 7 (G7) included samples from 31 seropositive patients from Brazil with chronic CD and the following clinical manifestations: asymptomatic ($n = 5$), cardiac ($n = 17$), digestive ($n = 2$), and cardiodigestive ($n = 7$). Thirteen patients were infected with TcII. Group 8 (G8) included samples from 75 seropositive patients from Argentina with chronic CD and the following clinical manifestations: asymptomatic ($n = 27$), cardiac ($n = 34$), digestive ($n = 1$), and cardiodigestive ($n = 13$). Fifty-one patients were infected with TcV or TcVI or combinations of TcII, TcV, and TcVI.

In addition, samples from 50 persons from Argentina with negative serology for *T. cruzi* were included as negative controls to address the specificity of the procedures.

DNA Extraction

The blood samples were obtained and immediately mixed with an equal volume of GE (GEB). After 48 to 72 hours at room temperature GEB samples were boiled for 15 minutes (except for G3 and G5 groups and seronegative samples) and stored at 4°C for DNA extraction and PCR analysis.

GEB samples were processed with the High Pure PCR Template Preparation kit (Roche Diagnostics Corp., Indianapolis, IN) as described in Duffy et al.[9] Those samples with cycle threshold (Ct) values lower than the Ct values for the most concentrated point of the standard curve were properly diluted in seronegative human blood treated with GE, and DNA extraction and qPCR procedures were repeated. To build the standard curves for quantification of parasitic loads, DNA from spiked blood samples were obtained in the same way as reported for the clinical samples. The DNA eluate was stored at −20°C until use in qPCR analysis.

Duplex Real-Time PCR Procedures

Two duplex qPCR procedures were compared, Satellite DNA (SatDNA) qPCR and kinetoplastid DNA (kDNA) qPCR assays. The former targets the satellite sequence of the nuclear genome of the parasite and the sequence of an internal amplification control (IAC) as described in Duffy et al,[9] and the latter is a modification of the method reported by Qvarnstrom et al[11] which targets the conserved region of the minicircle parasite sequences with the addition of primers and TaqMan probe for the IAC.[9] Both reactions were performed with 5 μL of re-suspended DNA, using FastStart Universal Probe Master Mix (Roche Diagnostics GmbHCorp., Mannheim, Germany) in a final volume of 20 μL.

Optimal cycling conditions for both qPCR assays were a first step of 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 58°C for 1 minute. The amplifications were performed using Rotor-Gene 6000 (Corbett Life Science, Cambridgeshire, United Kingdom) or ABI7500 (Applied Biosystems, Foster City, CA) devices.

Standard curves were plotted with 1/10 serial dilutions of total DNA obtained from a GEB-seronegative sample spiked with 10^5 parasite equivalents per milliliter of blood (par. eq./mL). TcId- and TcVI-DNA−based standard curves were used to quantify parasitic loads in G1a, G1b, G2, G3a, G4, and G5 and in G3b, G6, G7, and G8 samples, respectively.

Duplex Real-Time PCR Assays Performance

Terms

On the basis of the MICROVAL protocol,[12] the analytical validation of both qPCR methods included the following parameters. i) Selectivity is defined as a measure of the degree of response from target and nontarget microorganisms and comprises inclusivity and exclusivity. Inclusivity is the ability of an alternative method (each qPCR assay in this case) to detect the target pathogen from different strains (DTUs in this case), and exclusivity is the lack of response from closely related but nontarget strains (other trypanosomatids in this case). ii) Reportable range is a set of values of measurands for which the error of a measuring instrument is intended to lie within specified limits. iii) Limit of detection (LOD) is the smallest amount that the method can reliably detect to determine the presence or absence of an analyte. iv) Precision is the closeness of agreement between independent test/measurement results obtained under stipulated conditions. v) Limit of quantification (LOQ) is the smallest amount that the method can reliably measure quantitatively.

The above-mentioned parameters were evaluated in the framework of the international study as described in the sections below.

Inclusivity

Both qPCR assays were tested with genomic DNA obtained from a panel of *T. cruzi* stocks belonging to the six different DTUs, plus TcI stocks representative of three different TcI Spliced Leader Intergenic Region (SL-IR)−based groups (TcIa, TcId, and TcIe), in concentrations that ranged from 0.0625 to 10 fg/μL tested on duplicates: TcI [K98 (TcIa SL-IR−based group), G (TcId group), and SE9V (TcIe

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group) stocks][13–16]; TcII (Tu18 stock), TcIII (M5361 stock), TcIV (CanIII stock), TcV (PAH265 stock), and TcVI (CL-Brener stock).[17]

Exclusivity

Serial dilutions of purified DNAs from *Trypanosoma rangeli* and *Leishmania major*, *Leishmania mexicana* and *Leishmania amazonensis* that ranged from 0.1 fg/μL to 1000 pg/μL were assayed on duplicates by both qPCRs.

Reportable Range

A panel of GEB samples spiked with Sylvio X10 (TcId) and CL-Brener (TcVI) stocks, spanning 10^5 to 0.0625 par. eq./mL was prepared. After DNA purification, each dilution was amplified on triplicate by both qPCR assays. Assigned (theoretical) versus measured values were converted to Log_{10} par. eq./10 mL and plotted for linear regression analysis.

LOD

The LOD was calculated as the lowest parasitic load that gives ≥95% of qPCR detectable results, according to the Clinical and Laboratory Standards Institute guidelines.[18] It was measured from two panels of GEB samples spiked with the CL-Brener stock; one panel was boiled for 15 minutes before preparing serial dilutions,[7] and the other panel was diluted without prior boiling. For both panels, eight replicates from GEB dilutions that contained 0.125, 0.25, 0.5, and 1 par. eq./mL for SatDNA qPCR and 0.0625, 0.125, 0.25, and 0.5 par. eq./mL for kDNA qPCR were purified and amplified during five consecutive days. The LOD was determined by Probit regression analysis with Minitab 15 Statistical Software (Minitab Inc., State College, PA).

Precision

Precision experiments were performed with spiked GEB samples at concentrations of 0.5, 10, and 10^3 par. eq./mL (0.699, 2, and 4 Log_{10} par. eq./10 mL) for SatDNA qPCR and 0.25, 10, and 10^3 par. eq./mL (0.398, 2, and 4 Log_{10} par. eq./10 mL) for kDNA qPCR, assayed on duplicates during 20 consecutive experiments, one run per day, according to the Clinical and Laboratory Standards Institute guidelines.[19] The estimates of within-laboratory precision SDs (S_t) were calculated with the following formula: $S_t = [B^2 + (N-1)/N \times S_r^2]^{1/2}$, where B is the SD of the daily means and S_r is the estimate of repeatability SD (within-run precision).

LOQ

For both qPCR methods, the LOQ was derived from a 20% threshold value for the CV of measurements obtained in the precision experiments, as described in Schwarz et al.[20] Assuming an exponential decrease in CV, a curve for the relation between CV and Log_{10} par. eq./10 mL was fitted with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA).

Quality Controls for Analysis of Clinical Specimens

A negative control and two positive controls that contained different concentrations of *T. cruzi* DNA (namely a high-positive control and a low-positive control near the limit of detection) were included in every run as recommended.[6]

Internal Amplification Control

A pZErO-2 recombinant plasmid that contains an inserted sequence of *Arabidopsis thaliana* aquaporin was used as a heterologous extrinsic IAC.[8]

RNase P Assay

To check for DNA integrity, clinical samples were tested with TaqMan RNase P Control Reagents Kit (Applied Biosystems) in an ABI7500 Real-Time PCR device.

T. cruzi Genotyping

SatDNA qPCR quantifiable samples collected from laboratories that did not perform *T. cruzi* DTU typing (G1, G3, and G6) were genotyped with PCR-based strategies targeted to nuclear genomic markers, as described in Burgos et al.[21]

Evaluation of Parasitemia with Hemoculture

The 15 samples provided by the laboratory of Disciplina de Parasitología, Universidade Federal do Triângulo Mineiro (Uberaba, Brazil), were evaluated by hemoculture. The assay was performed according to the method described in Chiari et al.[22] Immediately after collection, 30 mL of blood was centrifuged at 4°C to remove plasma. The packed cells were washed by centrifugation at 4°C in liver infusion tryptose medium. The sedimented erythrocytes were resuspended in 30 mL of liver infusion tryptose medium and uniformly distributed in six test tubes. Cultures were maintained at 28°C and homogenized weekly. The culture was microscopically examined 30, 60, and 90 days after culture in 10-μL aliquots of suspension.

Statistical Analysis

The Cohen κ coefficient[23] was used to compare the clinical sensitivity of SatDNA and kDNA qPCRs for the detection of *T. cruzi* DNA in samples from chronic CD patients. The unpaired *t*-test was used to compare the means of parasitic loads of quantifiable samples from acute versus chronic CD patients and asymptomatic versus symptomatic chronic CD patients for both qPCR methods. In addition, nonparametric analysis of variance was used to compare the parasitic loads of quantifiable samples grouped according to their *T. cruzi* genotypes for each qPCR assay. Bland-Altman bias plot[6] was used to analyze the closeness of the agreement between the quantifiable results of both qPCR methods.

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Finally, the paired *t*-test was used to compare the means of IAC Ct values of both qPCR assays, and the Tukey criterion[24] was used to detect samples with outlier Ct values of IAC (Ct > 75th percentile + 1.5× interquartile distance of median Ct), which indicated PCR inhibition or material loss during sample DNA extractions.

Results

Analytical Validation of qPCR Methods

The analytical validation results obtained for both qPCR assays are shown in Table 1. For the inclusivity, qPCR methods were assayed with DNA from strains that represented the six *T. cruzi* DTUs, plus TcI stocks representative of three different TcI SL-IR−based groups (TcIa, TcId, and TcIe).

kDNA qPCR gave the same analytical sensitivity of 0.0625 fg/μL DNA, the lowest concentration tested, for all *T. cruzi* stocks analyzed. SatDNA qPCR gave an analytical sensitivity of 0.0625 fg/μL DNA for stocks representative of TcIa, TcII, TcIII, TcV, and TcVI; 0.25 fg/μL DNA for stocks belonging to TcId and TcIV; and 1 fg/μL DNA for the TcIe stock.

Exclusivity was assayed in *T. rangeli* and *Leishmania* spp. Both qPCR methods were nondetectable when up to 1000 pg/μL DNA, the highest concentration tested, from *Leishmania* stocks was analyzed. In the case of *T. rangeli*, 10 fg/μL DNA could be amplified by kDNA qPCR, whereas SatDNA qPCR required an input of at least 10 pg/μL *T. rangeli* DNA to obtain a detectable PCR result.

The reportable range was determined with 10 spiked GEB samples that contained serial dilutions of TcId- and TcVI-cultured epimastigotes. Linear regression analysis yielded the equation $y = 1.013x - 0.058$ ($R^2 = 0.992$) and $y = 1.001x - 0.005$ ($R^2 = 0.998$) for SatDNA qPCR, and $y = 0.813x + 0.824$ ($R^2 = 0.969$) and $y = 1.011x - 0.048$ ($R^2 = 0.984$) for kDNA qPCR and for TcId and TcVI representative stocks, respectively. Accordingly, the reportable range was from 10^5 to 1 par. eq./mL for TcId stock and from 10^5 to 0.25 par. eq./mL for TcVI stock, for both qPCR methods.

The LOD was determined for TcVI DTU. It was 0.16 par. eq./mL (95% CI, 0.13−0.24 par. eq./mL) and 0.23 par. eq./mL (95% CI, 0.18−0.35 par. eq./mL) for kDNA qPCR in boiled and nonboiled samples, respectively ($P = 0.013$). For SatDNA qPCR the LOD was 0.46 par. eq./mL (95% CI, 0.36−0.64 par. eq./mL) and 0.70 par. eq./mL (95% CI, 0.54−1.01 par. eq./mL) in boiled and nonboiled samples, respectively ($P = 0.044$). The LOD of kDNA qPCR was lower than that of SatDNA qPCR for both boiled ($P = 0.013$) and nonboiled samples ($P = 0.044$).

Estimates of precision were calculated for nonboiled GEB samples spiked with TcVI stock. The precision was higher for kDNA qPCR (CV = 31.98%) than for SatDNA qPCR (CV = 46.60%) at concentrations closer to the LOD, but it was higher for SatDNA qPCR (CV = 6.00% and 1.72%) than for kDNA qPCR (CV = 8.79% and 2.92%) for concentrations of 10 and 1000 par. eq./mL, respectively.

Table 1 Analytical Validation Results Obtained for qPCR Assays

Validation Parameters	SatDNA qPCR*	kDNA qPCR
Inclusivity (detectable qPCR), fg/μL		
TcIa	0.0625	0.0625
TcId	0.25	0.0625
TcIe	1	0.0625
TcII	0.0625	0.0625
TcIII	0.0625	0.0625
TcIV	0.25	0.0625
TcV	0.0625	0.0625
TcVI	0.0625	0.0625
Exclusivity (nondetectable qPCR), pg/μL		
T. rangeli	1	0.001
L. major	1000	1000
L. mexicana	1000	1000
L. amazonensis	1000	1000
Reportable range, par. eq./mL		
TcId	10^5−1	10^5−1
TcVI	10^5−0.25	10^5−0.25
Limit of detection, par. eq./mL		
Boiled	0.46	0.16
Nonboiled	0.70	0.23
Precision, CV %		
0.25 par. eq./mL	ND	31.98
0.5 par. eq./mL	46.60	ND
10 par. eq./mL	6.00	8.79
1000 par. eq./mL	1.72	2.92
Limit of quantification, par. eq./mL	1.53	0.90

*Data for SatDNA qPCR were taken from Duffy et al.[9]

kDNA, kinetoplastid DNA; ND, not done; par. eq./mL, parasite equivalents in 1 mL of blood; qPCR, quantitative real-time PCR; SatDNA, Satellite DNA.

The LOQ was derived from a 20% threshold value of the CVs obtained in the precision experiments. Linear least squares regression for the equation, $y = y_0 + axe^{-bx}$, resulted in the best fit ($R^2 = 1.0$) for both qPCRs. On the basis of the derived equation ($y = 1.61 + 157.75xe^{-1.81x}$ and $y = 1.79 + 43.39xe^{-0.91x}$), the absolute $LOQ_{20\%CV}$ was estimated in 1.53 par. eq./mL and 0.90 par. eq./mL for SatDNA and kDNA qPCRs, respectively.

Comparison of qPCR Results in Blood Samples

The performance of both qPCR methods was compared with DNA obtained from 156 GEB samples that covered different epidemiologic and clinical settings. In addition, 50 GEB samples from persons with negative serology for *T. cruzi* were tested. No amplification was detected for any of these negative controls with the use of both SatDNA and kDNA qPCR methods (clinical specificity, 100%).

The results obtained for the different patients' groups, including their geographic origin, routes of transmission, phase of CD, *T. cruzi* DTUs (only performed for samples with parasitic loads above SatDNA qPCR LOQ), qPCR positivity, and median parasitic loads, are summarized in Table 2.

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Table 2 Description of Clinical and Epidemiologic Data of the Different Groups of CD Patients Included in This Study, and Their Parasitic Loads Measured by SatDNA and kDNA qPCR Assays

								SatDNA qPCR			kDNA qPCR		
Group	Country	Transmission Route	CD Phase	Clinical Manifestation	*T. cruzi* DTU*	Boiled	Total (*n*)	Detectable qPCR, *n* (%)	Quantifiable samples, *n* (%)	Median [IQR] (Log_{10} par. eq./10 mL)	Detectable qPCR, *n* (%)	Quantifiable samples, *n* (%)	Median [IQR] (Log_{10} par. eq./10 mL)
G1a	Mexico	Unknown	Acute	Unknown	I	Yes	2	2 (100)	2 (100)	8.41 [8.32–8.50]	2 (100)	2 (100)	8.62 [8.55–8.69]
G1b			Chronic	Asymptomatic		Yes	2	2 (100)	1 (50)	2.48	2 (100)	1 (50)	2.74
G2	French Guiana	Oral	Acute	Cardiac	I	Yes	2	2 (100)	2 (100)	3.00 [2.64–3.35]	2 (100)	2 (100)	4.17 [3.88–4.46]
G3a	Bolivia	Oral	Acute	Unknown	IV	No	2	2 (100)	2 (100)	7.19 [7.18–7.20]	2 (100)	2 (100)	6.76 [6.69–6.83]
G3b		Vectorial or congenital	Chronic	Asymptomatic	V/VI	No	3	1 (33.3)	0 (0)		2 (66.7)	0 (0)	
G4	Venezuela	Oral	Acute	Unknown	I	Yes	5	5 (100)	4 (80)	3.13 [2.14–3.95]	5 (100)	5 (100)	2.50 [2.43–4.46]
G5	Colombia	Vectorial or congenital	Chronic	Asymptomatic	I	No	13	13 (100)	8 (61.5)	2.43 [2.15–2.66]	12 (92.3)	8 (61.5)	2.39 [2.03–2.92]
G6	Spain (Bolivian immigrants)	Vectorial or congenital	Chronic	Asymptomatic	V/VI	Yes	20	13 (65)	2 (10)	1.65 [1.63–1.66]	17 (85)	4 (20)	1.49 [1.10–1.91]
				Digestive		Yes	1	1 (100)	1 (100)	2.06	1 (100)	1 (100)	1.84
G7	Brazil	Unknown	Chronic	Asymptomatic	II	Yes	5	4 (80)	0 (0)		4 (80)	0 (0)	
				Cardiac		Yes	17	16 (94.1)	1 (5.9)	1.79	14 (82.4)	5 (29.4)	1.04 [1.01–1.23]
				Digestive		Yes	2	2 (100)	0 (0)		2 (100)	1 (50)	1.05
				Cardiodigestive		Yes	7	5 (71.4)	3 (42.9)	1.93 [1.58–2.06]	5 (71.4)	5 (71.4)	1.56 [1.09–2.03]
G8	Argentina	Unknown	Chronic	Asymptomatic	V/VI	Yes	27	22 (81.5)	7 (25.9)	1.86 [1.61–2.24]	21 (77.8)	11 (40.7)	1.57 [1.31–3.11]
				Cardiac		Yes	34	25 (73.5)	10 (29.4)	1.81 [1.42–2.58]	29 (85.3)	13 (38.2)	1.59 [1.40–2.09]
				Digestive		Yes	1	1 (100)	0 (0)		1 (100)	1 (100)	1.79
				Cardiodigestive		Yes	13	12 (92.3)	5 (38.5)	1.74 [1.49–1.74]	12 (92.3)	6 (46.2)	1.48 [1.37–2.14]

*Genotyping was only performed for samples with parasitic loads above SatDNA qPCR limit of quantification (1.53 par. eq./mL).

CD, Chagas disease; DTU, discrete typing unit; IQR, interquartile range; kDNA, kinetoplastid DNA; par. eq./10 mL, parasite equivalents in 10 mL of blood; qPCR, quantitative real-time PCR; SatDNA, Satellite DNA.

Acute CD

This group comprised 11 CD patients, 10 positives and 1 negative for serologic studies. All of them were qPCR detectable with the use of both SatDNA and kDNA qPCR methods (clinical sensitivity for acute CD, 100%). All cases also gave quantifiable parasitic loads by both qPCR methods, except one patient from Venezuela that was nonquantifiable with the use of SatDNA qPCR. The sample from the single seronegative patient from French Guiana presented 3.71 and 4.75 Log_{10} par. eq./10 mL for SatDNA and kDNA qPCRs, respectively. Most of these patients were infected by the oral route, and DNA from DTUs TcI or TcIV was detected in their samples. Only the patients from French Guiana presented cardiac symptoms.

Chronic CD

This group comprised 70 asymptomatic persons and 75 patients with cardiac and/or digestive syndromes. Except for the patients from Bolivia and Colombia who acquired the infection from vectors or congenitally, the transmission route in the remainder was unknown. Parasite genotyping revealed that the chronic CD patients were infected with all *T. cruzi* DTUs, except TcIII and TcIV.

For chronic CD patients, 117 and 122 of 145 samples were detectable with the use of SatDNA and kDNA qPCRs, clinical sensitivity equaled 80.69% and 84.14%, respectively; 113 of these samples were detectable by both qPCR methods, and 4 and 9 samples were detectable only by SatDNA or kDNA qPCRs, respectively; κ index was 0.691 (95% CI, 0.535–0.847). Moreover, 38 of 117 (32.48%) and 56 of 122 (45.90%) qPCR detectable samples were quantifiable with the use of SatDNA and kDNA qPCRs, respectively.

The details of the 13 samples with discordant qPCR results are shown in Table 3; because of the low parasitic loads of these samples, in each case the genotype was assumed to be that of the group to which the sample belonged.

The nine kDNA qPCR detectable and SatDNA qPCR nondetectable samples belonged to four cardiac patients from Argentina and five asymptomatic cases from Bolivia, four of them living in Spain, infected with TcV, TcVI, or combinations of TcII, TcV, and TcVI. The four samples that were SatDNA qPCR detectable but nondetectable with the use of kDNA qPCR included samples from two cardiac patients from Brazil infected with TcII, one Colombian asymptomatic case infected with TcI, and one Argentinean asymptomatic case infected with TcV, TcVI, or combinations of TcII, TcV, and TcVI. Only two of these samples were boiled, the Bolivian sample that was kDNA qPCR detectable and SatDNA qPCR nondetectable and the Colombian sample that was SatDNA qPCR detectable and kDNA qPCR nondetectable. The parasitic loads of all these samples were below the LOQ of the corresponding qPCR detecting method; moreover,

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Table 3 Discordant Findings Using SatDNA and kDNA qPCR Assays to Evaluate Clinical Samples during the International Study

Country	Sample ID	Clinical Manifestation	*T. cruzi* DTU*	Boiled	SatDNA qPCR Ct	SatDNA qPCR par. eq./mL	kDNA qPCR Ct	kDNA qPCR par. eq./mL
Colombia	CE4	Asymptomatic	I	No	37.01	NQ	ND	
Brazil	Fx230	Cardiac	II	Yes	34.04	NQ	ND	
	Fx2754				39.01	NQ	ND	
Bolivia	M4	Asymptomatic	V/VI	No	ND		35.38	NQ
Spain (Bolivian immigrants)	12,547	Asymptomatic	V/VI	Yes	ND		33.08	NQ
	19,592				ND		33.31	NQ
	27,259				ND		32.88	NQ
	34,749				ND		34.98	NQ
Argentina	PCH15	Asymptomatic	V/VI	Yes	39.69	NQ	ND	
	PCH43	Cardiac	V/VI	Yes	ND		35.07	NQ
	PCH58				ND		37.75	NQ
	437				ND		35.68	NQ
	919				ND		34.53	NQ

*The genotype of each sample was assumed to be that of the group to which the sample belonged.

Ct, cycle threshold; DTU, discrete typing unit; kDNA, kinetoplastid DNA; ND, nondetectable; NQ, nonquantifiable; par. eq./mL, parasite equivalents in 1 mL of blood; qPCR, quantitative real-time PCR; SatDNA, Satellite DNA.

all four SatDNA qPCR detectable and kDNA qPCR nondetectable and five of the nine kDNA qPCR detectable and SatDNA qPCR nondetectable samples presented parasitic loads below the LOD of the corresponding qPCR assay (Table 1).

Figure 1 illustrates the comparative analysis of parasitic loads from quantifiable clinical samples on the basis of the CD phase of patients (Figure 1A), and the clinical status (Figure 1B) and *T. cruzi* DTUs (Figure 1C) of chronic CD patients, obtained by each qPCR assay.

Acute CD patients had higher parasitic loads (median, 4.03; interquartile range, 2.72 to 7.20 Log_{10} par. eq./10 mL) than chronic CD patients (1.90; 1.61 to 2.46 Log_{10} par. eq./10 mL) with the use of SatDNA qPCR ($P = 0.0086$). Likewise, with the use of kDNA qPCR the acute CD patients also had higher parasitic loads (4.67; 3.05 to 6.76 Log_{10} par. eq./10 mL) than the chronic CD patients (1.68; 1.31 to 2.28 Log_{10} par. eq./10 mL) ($P = 0.0026$) (Figure 1A).

The comparative analysis of the parasitic loads of chronic CD patients according to their clinical status (Figure 1B) did not show any significant differences between the parasitic loads of asymptomatic persons [(2.17; 1.65 to 2.48 Log_{10} par. eq./10 mL) and (1.97; 1.34 to 2.77 Log_{10} par. eq./10 mL)] and symptomatic patients [(1.77; 1.45 to 2.13 Log_{10} par. eq./10 mL) and (1.56; 1.21 to 2.01 Log_{10} par. eq./10 mL)] either for SatDNA ($P = 0.2977$) or kDNA ($P = 0.1914$) qPCRs, respectively.

Similarly, no significant differences were observed between the parasitic loads from chronic CD patients infected with TcI (2.47; 2.30 to 2.58 Log_{10} par. eq./10 mL), TcII (1.86; 1.65 to 2.00 Log_{10} par. eq./10 mL), and TcV, TcVI, or combinations of TcII, TcV, and TcVI (1.74; 1.49 to 2.11 Log_{10} par. eq./10 mL), with SatDNA qPCR. However, with the use of kDNA qPCR, the patients infected with TcI had higher parasitic loads (2.55; 2.08 to 2.87 Log_{10} par. eq./10 mL) than patients infected with TcV, TcVI, or combinations of TcII, TcV, and TcVI (1.59; 1.33 to 2.11 Log_{10} par. eq./10 mL) (not significant), and with TcII (1.09; 1.03 to 1.78 Log_{10} par. eq./10 mL) ($P < 0.01$) (Figure 1C).

The degree of agreement between the quantifiable results obtained by both qPCR methods from clinical samples is represented in Figure 2 as a Bland-Altman bias (difference) plot. As shown, the mean bias was determined to be 0.33 Log_{10} par. eq./10 mL, indicating a systematic bias of 2.1-fold parasite equivalents per 10 mL between both methods. However, the bias was not statistically significant because the 95% CI (1.55 to −0.89 Log_{10} par. eq./10 mL), expressed in Figure 2 as bias ± 2 SD, contains zero (no difference).

Analysis of Exogenous and Endogenous Amplification Controls

The IAC amplification was used for quality control throughout the procedure, from the DNA extractions to qPCR assays. Higher Ct values of IAC were obtained for the clinical samples with the use of SatDNA qPCR (19.05; 18.53 to 19.41) than kDNA qPCR (18.90; 18.40 to 19.25) ($P < 0.0001$); threshold Cts for outlier values were 20.72 and 20.52, respectively. However, the same three samples with IAC Ct outliers (24.89, 21.00, and 21.49, and 24.74, 20.98, and 21.45) were identified for both SatDNA and kDNA qPCRs, respectively. These three samples were detectable for *T. cruzi* DNA by both qPCRs, but their parasitic loads were below the LOQ of the corresponding qPCR assay (Table 1); except for the one with the lowest Ct values of IAC (21.00 and 20.98) which presented a parasitic load of 0.94 par. eq./mL

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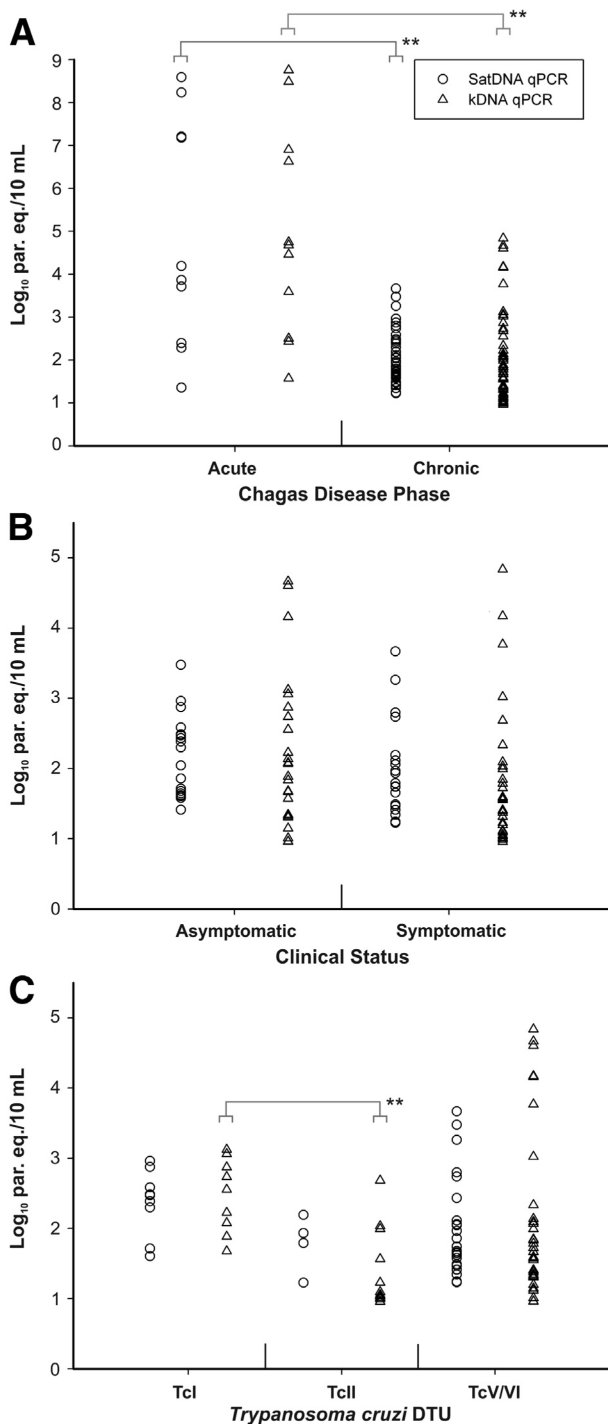

Figure 1 Comparative analysis of parasitic loads obtained by SatDNA (open circles) and kDNA (open triangles) qPCR assays for quantifiable samples from CD patients. Distribution of parasitic loads on the basis of the CD phase (**A**), the clinical status (**B**), and *T. cruzi* DTUs (**C**) of chronic CD patients. $^{**}P < 0.01$. CD, Chagas disease; DTU, discrete typing unit; kDNA, kinetoplastid DNA; par. eq./10 mL: parasite equivalents in 10 mL of blood; qPCR, quantitative real-time PCR; SatDNA, Satellite DNA.

(0.97 Log_{10} par. eq./10 mL) with the use of kDNA qPCR.

In addition, to evaluate DNA integrity of GEB samples, RNase P analysis was performed in a separate amplification reaction. All clinical samples were PCR detectable for this endogenous control with Ct values (22.32; 21.20 to 23.27) between cycle 19 and 27.

Comparison of qPCR Findings with Hemoculture

The parasitic loads of a subset of 15 GEB samples from Brazilian CD patients were compared with hemoculture results performed at the time the samples were collected (Table 4). Despite the time passed, an overall agreement was found between parasitic loads obtained by both qPCR methods and hemoculture results: the three samples with quantifiable parasitic loads corresponded to patients with five or six positive hemoculture results of a total of six replicates, whereas the three samples with nondetectable parasitic loads corresponded to patients with negative results by hemoculture assay, except one case with only one positive hemoculture result (Table 4).

Discussion

Analytical Validation of qPCR Methods in Different *T. cruzi* DTUs

This study presents the analytical validation and evaluation of two duplex qPCR methods on the basis of TaqMan probes designed for detection and quantification of *T. cruzi* DNA in human blood samples. The study was performed in the context of an international study organized in an attempt to establish standard operative procedures for quantification of parasitic loads in blood samples, using two methods ranked among the best ones by a previous qualitative PCR international study.[7]

This study involved the evaluation of DNA samples from parasite stocks representative of the different *T. cruzi* DTUs, including three distinct TcI SL-based groups. Analytical

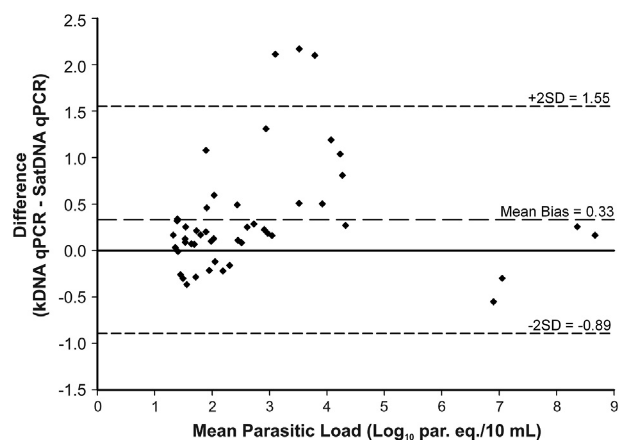

Figure 2 Bland-Altman bias (difference) plot analysis as a measure of the degree of agreement between the quantifiable results obtained by SatDNA and kDNA qPCR assays for samples from Chagas disease patients. kDNA, kinetoplastid DNA; par. eq./10 mL, parasite equivalents in 10 mL of blood; qPCR, quantitative real-time PCR; SatDNA, Satellite DNA.

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Table 4 Comparison of Hemoculture and qPCR Assays Results for a Subset of Fifteen Samples from Brazilian Chagas Disease Patients

Sample ID	Year	Clinical Manifestation	Hemoculture, positive/total	SatDNA qPCR, par. eq./mL	kDNA qPCR, par. eq./mL
87	1997	Cardiac	3/6	NQ	NQ
88		Cardiodigestive	4/6	NQ	1.24
86	1998	Cardiodigestive	0/6	ND	ND
93F	2000	Asymptomatic	0/6	ND	ND
90F		Asymptomatic	1/6	NQ	NQ
94F		Digestive	1/6	NQ	NQ
89F		Cardiodigestive	5/6	1.68	3.67
91F		Cardiodigestive	6/6	8.58	10.80
60	2002	Cardiac	1/6	ND	ND
01	2004	Asymptomatic	2/6	NQ	NQ
02	2006	Asymptomatic	6/6	NQ	NQ
72	2008	Cardiac	1/6	NQ	0.90
74		Cardiac	1/6	NQ	NQ
69		Cardiodigestive	3/6	NQ	0.98
66		Cardiodigestive	6/6	15.54	48.24

kDNA, kinetoplastid DNA; ND, nondetectable; NQ, nonquantifiable; par. eq./mL, parasite equivalents in 1 mL of blood; qPCR, quantitative real-time PCR; SatDNA, Satellite DNA.

validation was performed with seronegative blood spiked with known numbers of cultured epimastigotes and treated with GE buffer. Furthermore, blood samples from diverse clinical settings of different geographic regions and harboring different parasite DTUs were assayed. The parasitic loads of these samples were compared with the same DNA extraction protocols, qPCR amplification procedures, master mixes, PCR thermocyclers, and quality controls in the same laboratory.

Analytical sensitivity was more uniform among the different *T. cruzi* DTUs for kDNA qPCR than for SatDNA qPCR, because this latter method was less sensitive for some TcI and TcIV strains, indicative of lower gene dosage in their genomes.[8,25] Thus, in practice it would be advisable to construct standard qPCR curves with the use of regional strains representative of the prevailing DTUs in the affected population.

Parasitic Loads in Different Clinical Groups

As expected, parasitic loads in acute CD samples were higher than in chronic CD cases, reaching concentrations of up to 8 Log_{10} par. eq./10 mL for both qPCR methods. The four samples with the highest parasitic loads belonged to Mexican and Bolivian acute CD patients infected with TcI and TcIV, respectively. Accurate quantification of these samples was achieved after diluting them 1:10,000 in seronegative human GEB before doing the final DNA extractions and qPCR amplifications. One of the two acute CD samples from French Guiana belonged to a seronegative patient with suspicion of orally acquired *T. cruzi* infection on the basis of clinical and epidemiologic findings.[26] The qPCR positivity of this case points to the usefulness of molecular diagnostic methods for detecting acute CD cases before seroconversion.

Parasitic loads in chronic CD patients ranged from nonquantifiable to values of 3.67 and 4.84 Log_{10} par. eq./10 mL with the use of SatDNA and kDNA qPCRs, respectively. We observed differences in the parasitic loads between chronic CD patients infected with TcI (eight asymptomatic persons from Colombia plus one from Mexico) and TcII (11 symptomatic patients from Brazil), when they were analyzed with kDNA qPCR. This finding is in agreement with previous observations obtained by Moreira et al[27] with the use of a Sybr Green SatDNA qPCR assay in samples from Colombian and Brazilian symptomatic chronic CD patients recruited for the BENEFIT (Benznidazole Evaluation for Interrupting Trypanosomiasis) trial. Nevertheless, no differences were observed between the parasitic loads of asymptomatic and symptomatic chronic CD patients for the samples tested in our study. This is in agreement with previous observations that showed no correlation between *T. cruzi* parasitemia and clinical manifestations.[28,29] However, studies in the murine model reported that *T. cruzi* reinfections leading to an increase of parasitemia could be related to the variability and severity of the clinical course of CD.[30]

Bland-Altman analysis was performed to summarize the agreement between parasitic loads obtained by both qPCR methods by calculating the bias and by estimating the mean difference and the SD of the differences. It is also common to determine the limits of agreement, which are by convention set at the 95% CI of the difference between the methods, usually specified as bias $\pm$ 2 SD. If the 95% CI for the mean difference includes zero, such as in our study, there is statistically no evidence of bias.[6]

Some samples presented discordant qPCR results by either of the methods tested; kDNA qPCR detected more samples than SatDNA qPCR, which is reasonable because the former method had higher analytical sensitivity (lower LOD). Moreover, in most of the discordant samples the parasitic load was below the LOD, and in all cases below the LOQ, of the corresponding qPCR assay that gave detectable results.

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In this study, correlation between parasitic loads and frequency of positive hemocultures was also observed, strengthening the notion that detectable PCR results are indicative of live parasites.[31]

Use of Internal Amplification Controls

Previous studies for screening and quantification of parasitic loads of *T. cruzi* with the use of different real-time PCR approaches included a host DNA sequence, such as RNase P human gene, as an internal control.[8,10,27] This type of endogenous control is useful for qualitative purposes, as in the validation of the use of archival samples. In the present study, for instance, the RNase P assay was useful for checking the DNA integrity of GEB samples stored for >10 years. Nevertheless, we do not recommend the use of endogenous controls for quantitative purposes because the content of human blood cells can be highly variable, depending on the nutritional, metabolic, and immunologic status of the persons.[9] Therefore, a normalized amount of DNA of a plasmid that contains a heterologous sequence was added before DNA extractions and was used as an internal amplification control to monitor the whole procedure. Indeed, in our study, the IAC was useful to detect three samples with outlier Ct values.

Application of qPCR Methods in Different Scenarios of *T. cruzi* Infection

Comparison of the analytical parameters for both qPCR methods suggests that kDNA qPCR possesses higher sensitivity for detection and quantification of samples with low parasitic loads. However, in some Central and South American countries, such as Venezuela, Guatemala, Panama, Colombia, El Salvador, and some regions of Brazil, where *T. rangeli* might cause a false-positive diagnosis of *T. cruzi* infection,[32] SatDNA and not kDNA qPCR should be the qPCR-based method of choice (Table 1). Furthermore, qualitative and quantitative SatDNA real-time PCR approaches were recently used in clinical trials with new antiparasitic drugs and proved to be useful to detect treatment failure.[33,34]

Clinical sensitivities of both qPCR methods when tested in samples from chronic CD patients (80.69% and 84.14% for SatDNA and kDNA qPCRs, respectively) were similar to those obtained by the four best performing methods selected in a previous international PCR study for *T. cruzi* detection.[7] However, these sensitivities are not good enough for the application of PCR as confirmatory testing of blood donors or clinic patients who are serologically positive. Future prospective studies must be conducted to determine the optimal real-time PCR-based algorithm for diagnosis of *T. cruzi* infections in other epidemiologic and/or clinical scenarios, such as in early detection of congenital or oral transmission, and reactivation of infection in immunocompromised patients due to organ transplantation or HIV coinfection. Finally, the availability of these standardized and validated qPCR methods opens up new possibilities to monitoring patients in clinical trials with trypanocidal drugs,[27,33,34] contributing to improve the quality of life of CD patients.

Acknowledgments

We thank the Instituto de Biología y Medicina Experimental (IBYME), Instituto Nacional de Parasitología "Dr. Mario Fatala Chabén", and Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI) (Buenos Aires, Argentina) for supplying facilities and equipment and Life Technologies Argentina for providing the ABI StepOne Real-Time PCR device.

Samples from Mexico, French Guiana, Bolivia, Venezuela, Colombia, Spain, Brazil, and Argentina were provided by the Laboratorio Estatal de Salud Pública (Guerrero, Mexico), the Laboratoire Hospitalier et Universitaire-CH Andrée Rosemon (Cayenne, French Guiana), the Laboratorio de Parasitología y Biología Molecular, Instituto Nacional de Laboratorios en Salud (La Paz, Bolivia), the Instituto de Medicina Tropical, Universidad Central de Venezuela (Caracas, Venezuela), the Centro de Investigaciones en Microbiología y Parasitología Tropical, Universidad de los Andes (Bogota, Colombia), the Laboratorio de Parasitología Molecular, Pontificia Universidad Javeriana (Bogota, Colombia), and Centro Nacional de Microbiología, Instituto de Salud Carlos III (Madrid, Spain), the Laboratório de Biología Molecular e Doenças Endêmicas, Instituto Oswaldo Cruz/Fiocruz (Rio de Janeiro, Brazil), Universidade Federal do Triângulo Mineiro (Uberaba, Brazil), the Universidade Federal do Rio Grande do Norte (Natal, Brazil), the Instituto Nacional de Parasitología "Dr. Mario Fatala Chabén" (Buenos Aires, Argentina), and the Instituto de Patología Experimental, CONICET-Universidad Nacional de Salta (Salta, Argentina), respectively. Samples from seronegative persons were provided by Dr. Cecilia Irurtia (Hospital Posadas, Buenos Aires, Argentina).

We thank Drs. Patricio Diosque (IPE, CONICET-Universidad Nacional de Salta, Argentina) for PAH265, Tu18, CanIII, and M5631 strains; Aldo Solari (Universidad de Chile, Santiago, Chile) for SE9V isolate; Jose Franco Da Silveira (EPM, Sao Paulo, Brazil) for G isolate; Juan David Ramirez and Felipe Guhl (CIMPAT, Universidad de los Andes, Colombia) for *T. rangeli* DNA; and Alexandre Dasilva (CDC, Atlanta, GA) for *Leishmania* spp. DNAs.

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