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Parasitology

Comparison of four PCR methods for efficient detection of *Trypanosoma cruzi* in routine diagnostics

Peter Seiringer [a,b,1], Michael Pritsch [a,b,*,1], María Flores-Chavez [c], Edoardo Marchisio [d], Kerstin Helfrich [a], Carolin Mengele [a], Stefan Hohnerlein [a], Gisela Bretzel [a], Thomas Löscher [a], Michael Hoelscher [a,b], Nicole Berens-Riha [a]

[a] Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich (LMU), Leopoldstr. 5, 80802 Munich, Germany
[b] German Center for Infection Research (DZIF), partner site Munich, Munich, Germany
[c] Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo km 2, Madrid, Spain
[d] Dia.Pro Diagnostic BioProbes srl, Via Giosuè n°27, 20099 Sesto San Giovanni, Milan, Italy

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ABSTRACT

Due to increased migration, Chagas disease has become an international health problem. Reliable diagnosis of chronically infected people is crucial for prevention of non-vectorial transmission as well as treatment. This study compared four distinct PCR methods for detection of *Trypanosoma cruzi* DNA for the use in well-equipped routine diagnostic laboratories. DNA was extracted of *T. cruzi*-positive and negative patients' blood samples and cultured *T. cruzi*, *T. rangeli* as well as *Leishmania spp.* One conventional and two real-time PCR methods targeting a repetitive Sat-DNA sequence as well as one conventional PCR method targeting the variable region of the kDNA minicircle were compared for sensitivity, intra- and interassay precision, limit of detection, specificity and cross-reactivity. Considering the performance, costs and ease of use, an algorithm for PCR-diagnosis of patients with a positive serology for *T. cruzi* antibodies was developed.

1. Introduction

For decades after its discovery in 1909, Chagas disease (CD) was restricted to rural areas of South and Central America (Chagas, 1909; Dias et al., 2002; WHO, 2015). Urbanization, globalization and its facilitation of migration has transformed CD into an international health problem (Perez-Molina et al., 2012; Pinto Dias, 2013; Steverding, 2014; Vannucchi et al., 2014). In the last years, the number of infected individuals in previously non-endemic areas has significantly risen, amongst others in North America, the western Pacific region, and several European countries (Coura and Vinas, 2010; Jackson et al., 2010; Klein et al., 2012; Roca et al., 2011; WHO, 2010). Today, an estimated 6–7 million infections and 10,000 annual deaths are attributed to this life threatening disease worldwide (WHO, 2015).

CD is a chronic infectious disease caused by the protozoan flagellate *Trypanosoma cruzi* (*T. cruzi*). In endemic countries, CD is transmitted mainly through the contact with feces of different triatomine bugs. In non-endemic countries without vectorial transmission, the main risks for transmission are blood transfusions, organ transplantations and mother-to-child transmission, as women often migrate at young ages and later create families in their new homes (Cevallos and Hernandez, 2014; Perez-Ayala et al., 2011). CD presents itself in two phases: whereas the acute phase is dominated by unspecific or no symptoms at all, in the chronic phase cardiac and/or gastrointestinal symptoms can occur after a long asymptomatic interval (Rassi et al., 2010, 2012).

One of the first and very crucial steps in patient care is the diagnosis of the disease. Whereas serological methods remain the gold-standard for diagnosing CD because of their high sensitivity, performing a polymerase chain reaction (PCR) in addition can be important (Duarte et al., 2014; Godsel et al., 1995; Krautz et al., 1995; Krieger et al., 1992). Positive serological markers are only an indirect evidence of infection with *T. cruzi*, as they occur independently of the parasites' presence. The antibodies can persist in patients' blood for years even after successful treatment (Jackson et al., 2013; Sosa-Estani et al., 2009; Viotti et al., 2011). This has to be considered when monitoring treatment efficacy or quantifying parasite numbers in patients' blood (Avila et al., 1993). Other limiting factors of serology are the possible

* Corresponding author. Tel.: +49-8921803517; fax: +49-89336112.
E-mail addresses: peter.seiringer@lrz.uni-muenchen.de (P. Seiringer), pritsch@lrz.uni-muenchen.de (M. Pritsch), mflores@isciii.es (M. Flores-Chavez), edoardomarchisio@diapro.it (E. Marchisio), kerstin.helfrich@lrz.uni-muenchen.de (K. Helfrich), carolin.mengele@lrz.uni-muenchen.de (C. Mengele), stefan.hohnerlein@tum.de (S. Hohnerlein), bretzel@lrz.uni-muenchen.de (G. Bretzel), loescher@lrz.uni-muenchen.de (T. Löscher), hoelscher@lrz.uni-muenchen.de (M. Hoelscher), berens@lrz.uni-muenchen.de (N. Berens-Riha).
[1] Authors contributed equally.

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transfer of antibodies from mother to child (Mallimaci et al., 2010; Piron et al., 2007), recently acquired infections (Grauert et al., 1993), and the possible cross-reactivity with *Leishmania spp. (L. spp.)* or *Trypanosoma rangeli (T. rangeli)* (Gomes et al., 2009; Wincker et al., 1994a). In such cases and when obtaining doubtful or inconsistent serological results, PCR methods can be helpful (Marcon et al., 2002).

In 2014, researchers started an initiative to combat CD in Germany (http://chagas.info/) (Navarro et al., 2017). During the process of creating a suitable laboratory in Germany, the establishment of a quick, reliant and economic diagnostic algorithm was needed. As detailed in this study, four distinct PCR methods for detection of *T. cruzi* in human blood were intensely evaluated. The comparison of results led to the first two officially accredited PCR methods for the detection of *T. cruzi* in a German routine laboratory.

2. Materials and methods

2.1. Samples

Samples consisted of 50 human EDTA blood samples that were previously tested positive by ELISA, IFAT and PCR for *T. cruzi* in the Department of Parasitology, National Microbiology Centre, Instituto de Salud Carlos III, Madrid, Spain (ISCIII) during 2010–2013. The PCR was similar to method A described below. More detailed information on the patients is shown in Table 3. None of them have received treatment prior to this study. Further, three negatively tested human EDTA-blood samples for *T. cruzi* and *L. spp*. as well as cultured epimastigotes of *T. cruzi* (DM28) (Grisard et al., 2014), *T. rangeli* and *T. brucei* (patient material, not further characterized) were used. Two *T. cruzi* negative blood samples were spiked with the above mentioned culture material. Additionally, *L. tropica*-, *L. donovani*-, *L. infantum*-, *L. braziliensis*- and *L. major*-positive patient samples were taken from symptomatic outpatients who were treated at the Division of Infectious Diseases and Tropical Medicine (DITM), Medical Center of the University of Munich (LMU), during 2007–2013. All human samples were taken from patients that had agreed on the use of their anonymized blood samples for research purposes. Ethical approval was obtained from the Ethical Committee of the Faculty of Medicine of the Ludwig-Maximilians-University (LMU) in Munich, Germany. All data were processed anonymously. Data were transferred to STATA, version 14, and analysis was performed. Linear regression models were used for a multivariate analysis of continuous variables. Comparison between two groups was calculated by Wilcoxon rank sum test for continuous variables and the χ^2 test or Fisher's exact test for categorical variables. Results were considered significant if p-values were <0.05.

2.2. DNA purification

Patients' blood samples were treated with a mixture of 6 M guanidine hydrochloride, 0.2 M EDTA, pH 8.00 (guanidine-EDTA) in the ratio of 1:1 and left incubating for at least 12 hours as previously described (Avila et al., 1991; Britto et al., 1993; Duffy et al., 2013; Wincker et al., 1994b). Afterwards, the samples were boiled for 15 minutes, which partially disrupts the kDNA minicircle (Virreira et al., 2003). DNA extraction was carried out using the High Pure PCR Template Preparation kit (Roche Diagnostics Corp., Indianapolis, IN, USA) as described in the manufacturers' instructions. The purified DNA template was stored at ≤−20 °C. DNA extraction of cultured material was performed as described in the manufacturers' instructions without pre-treatment with guanidine hydrochloride.

2.3. DNA amplification

After extensive literature research and the recommendations of ISCIII, two conventional and two real-time PCR methods were elected for closer evaluation (Norman et al., 2011; Piron et al., 2007; Schijman et al., 2011): Two were conventional PCR methods, method A recommended by the ISCIII and method B by Schijman et al. (2011). Primers for method A target the variable region of the kDNA minicircle (Wincker et al., 1994a) and for method B a repetitive Sat-DNA sequence (Cummings and Tarleton, 2003) of the *T. cruzi* genome. Methods C and D were real-time PCR tests, both targeting repetitive Sat-DNA sequences. Method C was amongst the best performing methods in the work of Schijman et al. (2011), method D was a commercial kit (Dia.Pro - Diagnostic Bioprobes Srl, Sesto San Giovanni, Italy). For methods A and B the Professional Standard thermal cycler (Biometra GmbH, Göttingen, Germany) was used, for methods C and D the CFX C1000 Real-Time thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers are listed in Table 1. Amplification was performed as detailed below:

Method A: The PCR mix contained 1× AmpliTaq Gold Buffer, 2 mM $MgCl_2$, 0.33 μM of each of the kDNA specific primers 121 (Sturm et al., 1989), and 122 (Wincker et al., 1994a, 1994b), 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 2.5 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 10 μl template DNA and water, adding up to a final volume of 75 μl. The ~330 base pair (bp) fragment was amplified under the following conditions: 5 min at 95 °C; 35× (1 min at 94 °C, 1 min at 64 °C, 1 min at 72 °C); 10 min at 72 °C.

Method B: The PCR mix contained 1× AmpliTaq Gold Buffer, 3 mM $MgCl_2$, 0.5 μM of each of the Sat-DNA specific primers TczF and TczR (Cummings and Tarleton, 2003), 0.25 mM dNTPs, 1.5 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 10 μl template DNA and water, adding up to a final volume of 60 μl. The ~182 bp fragment was amplified under the following conditions: 3 min at 94 °C; 40× (45 s at 94 °C, 1 min at 68 °C, 1 min at 72 °C); 10 min at 72 °C (Schijman et al., 2011). PCR products for methods A and B were analyzed by electrophoresis in a 2% agarose gel in 1× TAE buffer stained with GelRed (Biotium, Hayward, CA, USA).

Method C: The PCR mix contained 1× FastStart Universal Probe Master (ROX) (Roche Diagnostics Corp., Indiana, USA), 0.75 μM of each of the Sat-DNA specific primers cruzi1, cruzi2 and TaqMan probe cruzi 3, 2 μl template DNA and water, adding up to a final volume of 20 μl. Cyling conditions of the ~166 bp fragment were as follows: 95 °C for 15 min, 45× (95 °C for 10 s, 54 °C for 1 min). Fluorescence was measured at the end of each cycle at 54 °C (Schijman et al., 2011).

Method D was performed with the commercially available kit TCRUZIDNA.CE (Diagnostic Bioprobes Srl, Sesto San Giovanni, Italy) following the manufacturer's instructions. Data of method C and D were analyzed with CFX Manager Software Version 2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The threshold was set to 50 relative fluorescent units (RFU) and thus sufficiently low to be within the exponential growth region of the amplification curve.

2.4. Controls

Adequate positive and negative controls were included in each run to detect possible contamination. After each DNA purification, the negative extraction control (water) was examined.

2.5. Selected definitions

The following terms were used to evaluate the performance of each PCR method:

Intra-assay precision (Intra). To evaluate the intra-assay precision, one known positive and one known negative sample were tested three times in one run on the first day of evaluation.

Inter-assay precision (Inter 1 and Inter 2). To evaluate the inter-assay precision, the same positive and negative samples used to determine the intra-assay precision were tested once on the second day (Inter 1) and once on the third day (Inter 2) of evaluation.

Limit of detection (LOD). After DNA amplification of cultured *T. cruzi*-strain DM28 as described above for methods A, B, C, and D, an electrophoresis of the amplicons in a 2% agarose gel in 1× TAE buffer stained

Table 1
Primers used in the study.

Method	PCR	Target	Primer	Cycles	Reference
A	conventional	kDNA	121: 5′-AAA TAA TGT ACG GGK GAG ATG CAT GA-3′(K = T or G) 122: 5′-GGT TCG ATT GGG GTT GGT GTA ATA TA-3′	35	(Sturm et al., 1989; Telleria et al., 2006)
B	conventional	Sat-DNA	TczF: 5′-GCT CTT GCC CAC AMG GGT GC-3′ (M = A or C) [modified] TczR: 5′-CCA AGC AGC GGA TAG TTC AGG-3′ [modified]	40	(Cummings and Tarleton, 2003)
C	Real-time	Sat-DNA	cruzi1: 5′-AST CGG CTG ATC GTT TTC GA-3′ (S = G or C) cruzi2: 5′-AAT TCC TCC AAG CAG CGG ATA-3′ cruzi3 (TaqMan®-Probe): 5′-6-FAM-CAC ACA CTG GAC ACC AA-BBQ quencher-3'	45	(Schijman et al., 2011)
D	Real-time	Sat-DNA	commercial Kit (DiaPro)	40	

with GelRed (Biotium, Hayward, CA, USA) was performed. Each visible *T. cruzi*-specific band was cut out under ultraviolet light and purified. Although two target sequences of different lengths were amplified in method B, we only used the stronger band with a length of 182 bp for LOD-determination. After purification of the amplicons, the DNA content was quantified using Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). Ten 1:10 dilutions with sterile water were prepared. Amplification was performed for methods A-D, using the dilutions as template DNA (Beissner et al., 2012). LOD was defined as the minimal concentration, in which at least 95% of the analyzed samples were detected (Bustin et al., 2009).

Cross-reactivity (*CR*). Possible cross-reactivity of non-target DNA was assessed by testing healthy adults' blood samples spiked with *T. rangeli* and *T. brucei* and the samples of patients infected with *L. tropica-, L. donovani-, L. infantum-, L. braziliensis-* and *L. major*.

Matrix (*Mtx*). To evaluate the presence of inhibiting factors, one known negative sample of a healthy person was spiked with culture material of *T. cruzi*-strain DM28 (Grisard et al., 2014) and processed as the other blood samples. For matrix evaluation, the spiked blood sample and the same, non-spiked sample were tested by using each method.

3. Results and discussion

Out of the 50 previously positive tested samples (by serology and PCR), 44 samples were detected by all four methods (Table 3). Method A detected all 50 samples, which is not surprising, as all samples have previously been tested positive by a protocol similar to method A. This selection bias was considered during analysis of data. Method A, which is known to be highly sensitive (Wincker et al., 1994a), was used as a reference for sensitivity of methods B, C, and D. Additionally, sensitivity of methods B, C, and D can be compared amongst each other, as protocols and targets were different to those used during external examination and pre-selection of samples. All other tests conducted in this study apply for methods A, B, C, and D because preconditions were equal and not biased.

Thus, compared to method A, method D showed the highest sensitivity (98%), followed by method B (94%). Method C, detecting only 45 out of 50 positive samples, showed lower sensitivity (90%).

There was no previously positive tested sample, which has not been detected by at least one of the four methods. There was one sample, which could only be detected by reference method A (P23). Methods B, C, and D failed to amplify DNA in this sample. One sample (P19) was detected by method C only, aside from reference method A. All other samples were detected by at least two of the methods B, C, or D.

Median age of the patient was 36.5 years (IQR 30.0; 47.5); 74% of the patients were female. Most patients (76%) originated from Bolivia. The rest were from other South American countries, Spain or unknown origin. All patients were in the chronic phase of CD when blood samples were taken, three were classified as suffering from recent chronic infection (Table 3). There was one patient with cardiac, two patients with gastrointestinal, and one patient with both cardiac and gastrointestinal symptoms. One patient presented himself with neuropathy which has not been further characterized. Patients younger than 15 years of age were considered to suffer from recent chronic CD, as described before (Bonney, 2014). There was no evidence of a congenital transmission, although it can hypothetically be assumed for the only two patients from Spain, P17 and P21, aged 2 and 3 years, respectively. Their Cq-values in real-time PCR were significantly below average (C: $P = 0.032$; D: $P = 0.018$), the intensity of DNA bands in conventional PCR was slightly above average (A + B: $P > 0.05$), indicating an elevated parasitic load. It has been shown before that younger pediatric patients with congenital CD have higher parasitic loads than older pediatric patients (Duffy et al., 2009).

There was no association between the intensity of DNA bands or Cq values with patients' symptoms, country of origin or sex. However, there was a significant correlation between intensity of DNA bands or Cq values and collection date of the samples. The older the sample

Table 2
Performance of PCR methods.

	Conventional						Real-Time					
	Method A			Method B			Method C			Method D		
Primers	121–122			TczF-TczR			cruzi1-cruzi2			Dia.Pro kit		
Target	kDNA			Sat-DNA			Sat-DNA			Sat-DNA		
Intra	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3
	pos	pos	pos	pos	pos	pos	Cq = 38.72	Cq = 37.02	Cq = 35.4	Cq = 30.06	Cq = 28.8	Cq = 30.08
Inter 1	pos			pos			Cq = 36.66			Cq = 29.98		
Inter 2	pos			pos			Cq = 36.55			Cq = 29.81		
Sens (*n = 50*)	50/50 pos			47/50 pos			45/50 pos			49/50 pos		
LOD	relative	absolute		relative	absolute		relative	absolute		relative	absolute	
	40 copies/µl	400 copies		40 copies/µl	400 copies		20 copies/µl	40 copies		2 copies/µl	10 copies	
Spec (*n = 3*)	3/3 neg			3/3 neg			3/3 neg			3/3 neg		
CR (*n = 7*)	1/7 pos			1/7 pos			1/7 pos			1/7 pos		
Mtx	pos			pos			pos			pos		

Summary of the results of methods A-D. Intra = Intraassay; Inter 1 = Interassay 1, Inter 2 = Interassay 2; Sens = Sensitivity; LOD = Limit of detection; Spec = Specificity; CR = Cross-reactivity; Mtx = Matrix effect; pos = positive assay; x/y pos = x of y samples tested positively; x/y neg = x of y samples tested negatively; copies = target copies; Cq = Cq (quantification cycle)-value.

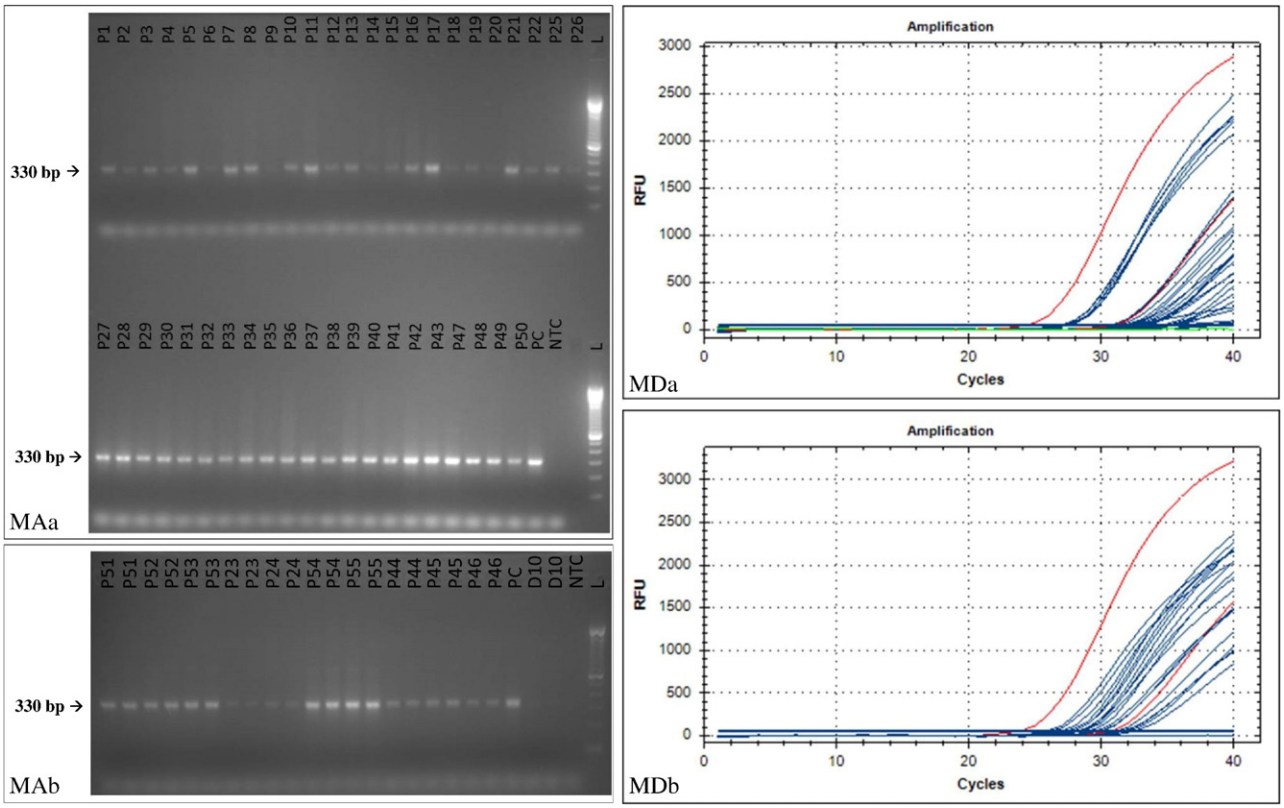

Fig. 1. Sensitivity assay. Sensitivity assay with methods A (MAa + MAb) and D (MDa + MDb). 45 (MAa) and 10 (MAb) positive samples were examined with method A. P51–55 were excluded in analysis due to lack of material for other methods. 30 (MDa) and 20 (MDb) positive samples were examined with method D. Protocols are described in *Materials and Methods*. In method D, the red graphs show fluorescence of high- and low positive controls, the green graph of no-template control (NTC), the blue graph of patients' samples. P1–55 = patients' samples; PC = positive control (*T. cruzi-strain* Dm28); D10 = *T. cruzi-strain* Dm28 in dilution 10^{-10}; NTC = No Template Control = Mastermix devoid of DNA; L = DNA ladder.

was, the lower the intensity and the higher the Cq value (A, B, C, D: $P < 0.001$, corrected for age, gender, symptoms and origin).

Potentially cross reactive agents (*L. tropica*, *L. donovani*, *L. infantum*, *L. braziliensis*, *L. major*, *T. rangeli* and *T. brucei*) showed similar results in all four methods: The *T. cruzi*-negative blood sample, which was spiked with a high concentration of *T. rangeli* (10^6 parasites/ml), was amplified by all four methods and showed a positive result with bands of similar length to those of *T. cruzi* (method A and method B). These findings have also been reported elsewhere (Ramirez et al., 2015). The other analytes did not amplify in any of the methods.

The LOD was lowest in real-time PCR method D. A total of 10 copies per run were still amplified in 10 out of 10 wells. For methods A, B and C, absolute LOD values were higher, 400 copies, 400 copies and 40 copies per reaction, respectively. It should be noted that method C still amplified 9 out of 10 samples with 4 copies per reaction.

Several studies about molecular diagnostics of CD in humans have been published (Britto et al., 1995; Cummings and Tarleton, 2003; Gilber et al., 2013; Norman et al., 2011; Piron et al., 2007). The recommendations of the ISCIII in Spain, which has a lot of experience in the field of CD-diagnostics (Flores-Chávez et al., 2007, 2010; Norman et al., 2011), and the work of Schijman et al. were milestones in this field (Schijman et al., 2011). The latter brings together several different PCR methods with various targets and allows an excellent overview over the PCR tests for detection of *T. cruzi* conducted worldwide at the moment. Some of its best performing methods (LbD3, LbF1, LbQ) were chosen and slightly adapted to the conditions at the Division of Infectious Diseases and Tropical Medicine in Munich. Method A of the present paper closely corresponds to test LbQ, method B to test LbD3 and method C to test LbF1.

Comparability of sensitivity is limited as all selected samples in the present evaluation had been tested positive not only by serology but also by PCR, using a similar protocol to method A before. However, the good performance of these tests shown in the study of Schijman et al. (2011) in terms of sensitivity (83.3–94.4%) and specificity (85–95%) could be confirmed in the present work, where high values for sensitivity and specificity were found (Table 2, Fig. 1). Also Gilber et al. (2013) found high sensitivity (76.4%) and specificity (100%) for a conventional PCR assay with similar primers to those of method B.

On the contrary, Duarte et al. (2014) reported low sensitivity (51% and 22%) and high specificity (100% and 100%) conducting similar protocols to those of methods A and B of the present study. The indicated lower sensitivity might be caused by misinterpretation of patient's clinical characteristics leading to a selection bias, the use of buffy coat and different reagents for analysis, the presence of inhibiting factors in the PCR, or the conduction of a simpler extraction method. Piron et al. (2007) found a sensitivity of 41% and a specificity of 100%, examining seropositive patients' samples by real-time PCR, using a similar protocol to that of method C of the present work.

Cummings and Tarleton (2003), using similar primers to those of methods A and B, though performing a Sybr Green-based quantitative real-time PCR assay, reported a lower LOD with primers targeting Sat-DNA than kDNA. These findings could not be confirmed, as conventional methods A and B showed similar LOD values (400 target copies/reaction). Real-time methods C and D, both using Sat-DNA primers and probes, showed significantly lower LOD values than conventional assays, 40 target copies/reaction and 10 target copies/reaction, respectively. Ramirez et al. (2015) reported a lower LOD, thus higher analytical sensitivity, of kDNA PCR compared to Sat-DNA PCR tests for *T. cruzi* VI. In the present work, the conventional kDNA PCR showed the highest sensitivity but no difference concerning the LOD compared to conventional Sat-DNA PCR and higher LOD compared to real-time Sat-DNA PCR methods.

Amplification in all 4 methods could be shown in the *T. cruzi*-negative blood sample, which was spiked with a high concentration of *T. rangeli* (10^6 parasites/ml). Bands in methods A and B were of similar length to those of *T. cruzi*-amplicons. *T. rangeli*-specific bands of 760 bp

Table 3
Specific results and patient data.

Sample	Age	Sex	Country of origin	Phase of disease	Symptoms	Year	Methods			
							A	B	C	D
							Primers			
							121–122	TczF-TczR	cruzi1-cruzi2	Dia.Pro
P1	36	M	BOL	chr	none	2010	++	++	34.17	32.27
P2	54	F	BOL	chr	gas	2010	+	+	35.51	35.50
P3	29	F	BOL	chr	none	2010	++	+	32.27	32.37
P4	26	F	BOL	chr	none	2011	+	++	35.03	33.53
P5	52	F	BOL	chr	gas	2011	+++	++	30.25	30.70
P6	40	M	CHL	chr	none	2011	(+)	+	N/A	34.63
P7	59	M	BOL	chr	neu	2011	+++	++	34.41	35.90
P8	25	F	BOL	chr	none	2011	+++	++	32.42	30.79
P9	30	F	END/unknown	chr	none	2011	(+)	+	36.59	33.13
P10	32	F	BOL	chr	none	2011	++	+	34.44	31.72
P11	30	F	BOL	chr	none	2011	+++	++	31.46	29.26
P12	31	F	BOL	chr	none	2011	++	++	34.91	31.35
P13	51	M	BOL	chr	none	2011	++	+	35.25	33.24
P14	31	F	END/unknown	chr	none	2011	+	−	39.37	33.47
P15	35	F	BOL	chr	none	2011	+	+	35.20	33.28
P16	38	M	BOL	chr	none	2011	++	++	33.31	32.14
P17	2	M	ESP	rec chr	none	2011	+++	++	28.12	25.56
P18	57	F	END/unknown	chr	none	2011	+	+	36.16	31.69
P19	50	F	BOL	chr	none	2011	+	−	N/A	39.39
P20	38	M	BOL	chr	none	2011	(+)	(+)	N/A	37.99
P21	3	M	ESP	rec chr	none	2011	+++	++	28.46	25.95
P22	38	F	BOL	chr	none	2011	+	++	N/A	35.35
P23	54	M	BOL	chr	none	2013	+	−	N/A	N/A
P24	37	F	BOL	chr	none	2013	+	++	35.12	31.43
P25	40	F	BOL	chr	none	2013	++	++	35.78	33.25
P26	31	F	BOL	chr	none	2013	(+)	++	33.97	35.13
P27	35	F	BOL	chr	none	2013	++++	++	28.87	32.24
P28	14	M	BOL	rec chr	none	2013	++++	++	29.48	30.95
P29	47	F	BOL	chr	none	2013	+++	++	30.22	27.51
P30	38	F	END/unknown	chr	none	2013	+++	++	29.11	26.66
P31	38	F	BOL	chr	none	2013	+++	++	30.57	31.22
P32	38	F	BOL	chr	none	2013	+++	++	30.47	28.26
P33	34	F	BOL	chr	none	2013	+++	++	27.77	27.43
P34	30	F	END/unknown	chr	none	2013	+++	+	30.45	28.02
P35	28	F	END/unknown	chr	none	2013	+++	++	30.50	27.96
P36	26	F	BOL	chr	none	2013	+++	+	30.05	28.13
P37	23	F	BOL	chr	none	2013	++++	++	28.15	27.02
P38	29	F	END/unknown	chr	none	2013	+++	++	35.10	35.34
P39	49	F	BOL	chr	none	2013	++++	++	29.44	27.18
P40	46	F	BOL	chr	car	2013	++++	++	31.26	32.98
P41	35	M	BOL	chr	car, gas	2013	++++	++	31.24	29.00
P42	49	F	BOL	chr	none	2013	++++	++	28.45	27.09
P43	38	F	BOL	chr	none	2013	++++	+++	29.06	27.42
P44	35	F	BOL	chr	none	2013	++	++	31.65	30.95
P45	40	F	PRY	chr	none	2013	++	++	31.22	29.73
P46	34	F	BOL	chr	none	2013	++	++	31.18	29.62
P47	50	M	BOL	chr	none	2013	++++	++	30.08	27.25
P48	31	F	BOL	chr	none	2013	++++	++	34.08	32.68
P49	55	M	END/unknown	chr	none	2013	++++	+++	29.87	27.48
P50	51	F	BOL	chr	none	2013	+++	+++	29.56	27.50
						mean 2010	1.67	1.33	33.98	33.38
						mean 2011	1.76	1.39	33.69	32.58
						mean 2013	3.02	1.96	30.84	29.61
						mean all	2.46	1.71	32.00	30.99

For conventional PCR tests (method A and method B), the results were specified according to the intensity of the visible DNA bands after exposure to ultraviolet (UV) light as follows: not visible -, just visible (+), moderately visible +, visible ++, strongly visible +++, very strongly visible ++++. For real-time Methods 3 and 4, quantification cycle (Cq)-values were specified, according to CFX Manager Software Version 2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Mean values are indicated as the average number of + − signs, whereas (+) = 0.5, + = 1, ++ = 2, +++ = 3, ++++ = 4 for methods 1 and 2 and the average Cq-value for methods 3 and 4. Mean values were calculated for each year of sample acquisition separately and once cumulative for all samples, for each PCR method. Patients' age indicated in years. Patients P8 and P35 were pregnant at the time of venipuncture. P1–50 = patients' samples; F = Female; M = Male; BOL = Bolivia; CHL = Chile; END/unknown = if exact country of origin was unknown, patient was assumed to be from the endemic region (ranging from the south of the United States to the south of Argentina); ESP = Spain; PRY = Paraguay; chr = chronic (>14 years of age); rec chr = recent chronic (0–14 years of age); car = cardiomyopathy; gas = gastrointestinal disease; neu = neuropathy; year = year in which blood sample of patient has been taken; N/A = not applicable.

and 380 bp were not amplified, although reported before (Botero et al., 2010; de Sousa et al., 2008).

Gilber et al. (2013) report an amplification of *T. rangeli*, *L. braziliensis* and *L. amazonensis* in a conventional PCR assay, using similar primers to those of method B.

These data suggest that a differentiation between *T. cruzi* and *T. rangeli* is impossible by any of the four analyzed methods in areas where both parasites are co-circulating. Rather should diagnostic results be evaluated together with the origin and clinical symptoms of the patient.

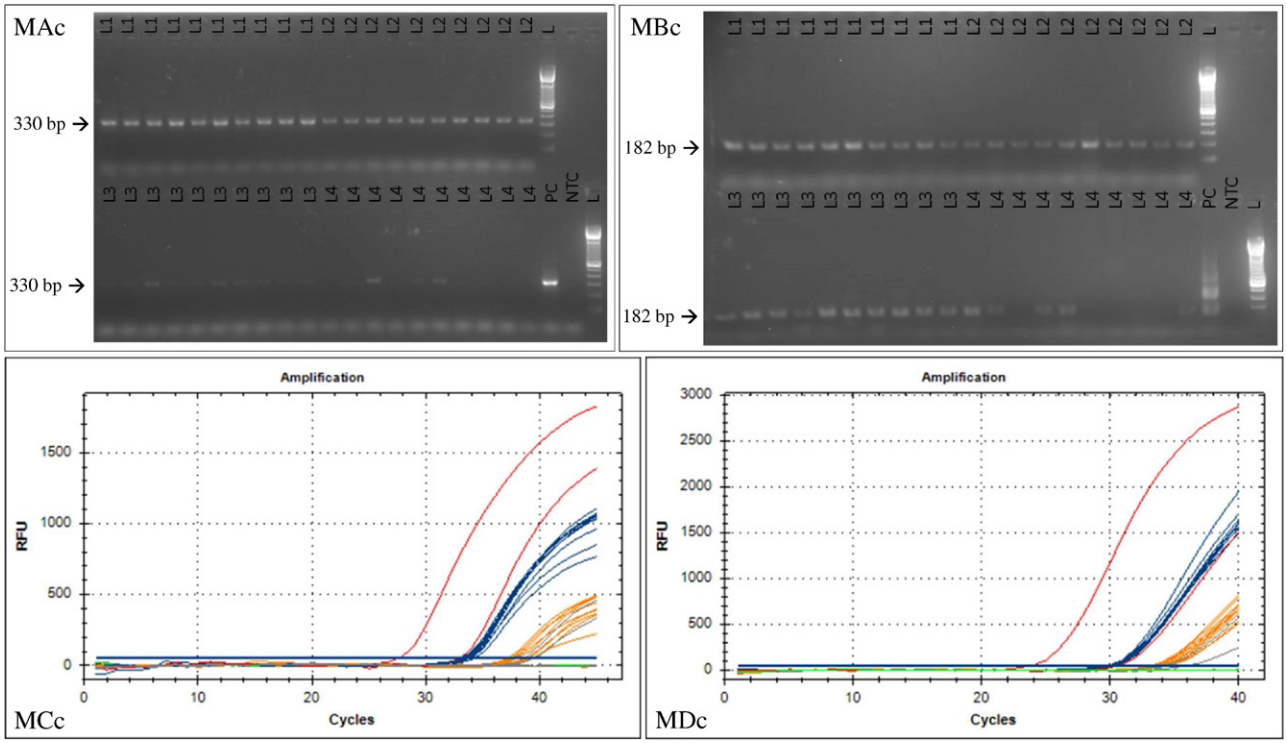

Fig. 2. Limit of detection. Limit of detection for method A (MAc), method B (MBc), method C (MCc) and method D (MDc). Assay described in Terms. MAc + MBc: L1 = 4000 target copies/µl; L2 = 400 target copies/µl; L3 = 40 target copies/µl; L4 = 4 target copies/µl; MCc + MDc: blue graphs = 20 target copies/µl; orange graphs = 2 target copies/µl, gray graphs = 0.2 target copies/µl; red graphs = fluorescence of high- and low positive controls; green graph = No Template Control = Mastermix devoid of DNA; L = DNA Ladder.

The aim of this work was to find a fast and reliant algorithm for molecular-biological diagnostics of CD in routine laboratories. As expected, all analyzed methods performed well. Though, considering the performance, the cost and the ease of use of each test, there are slight differences.

The biggest variability between the tests was observed in sensitivity and LOD assays, and the costs. Again, it has to be pointed out that method A (sensitivity = 100%) was used as a reference method for the sensitivity assay. Sensitivity of methods B, C, and D in comparison to method A ranged from 90% to 98%. Out of the 50 previously positive tested samples (by serology and PCR), there were 44 samples, which have been detected by all methods (B, C, D). This emphasizes the good performance of all evaluated methods. One sample detected by method A did not show amplification in method D. This is surprising, as the analytical LOD of method D was lower than the LOD of method A (Table 2, Fig. 2). One of the reasons might be a low number of Sat-DNA copies in these samples, as the number of Sat-DNA target copies vary highly depending on the *T. cruzi* lineage (Duffy et al., 2009). Whereas method D targets Sat-DNA, method A targets kDNA minicircles. Another explanation might be lineage differences due to Sat-DNA polymorphisms.

As copy numbers of targets used in methods A, B, C, and D and DNA content per parasite vary highly depending on the lineage, the copy numbers indicated in the LOD assay were not converted into corresponding parasite cells. The absolute DNA content, which also depends on the lineage of the parasite, is enumerated as 0.12–0.33 pg per cell (Duffy et al., 2009; Kooy et al., 1989; Lanar et al., 1981; McDaniel and Dvorak, 1993; Santos et al., 1997; Thompson and Dvorak, 1989). Specifications for kDNA range from 5,000 to 30,000 minicircles per parasite (Gonzalez et al., 1984; Degrave et al., 1988; Sturm et al., 1989; Britto et al., 1993; Cummings and Tarleton, 2003; Ramirez et al., 2009), which sums up to 20,000–120,000 copies per parasite of the variable region of the minicircle used as a target in Method A, considering the fact that there are four copies of the target region per minicircle (Avila et al., 1990; Degrave et al., 1988; Sturm et al., 1989). Specifications for Sat-DNA copies targeted by PCR methods in this study are highly dependent on the lineage of the parasite and range from about 10,000 to more than 120,000 copies per cell (Duffy et al., 2009; Gonzalez et al., 1984; Martins et al., 2008; Moser et al., 1989; Ramirez et al., 2009).

Costs of consumables are significantly cheaper for conventional methods A and B than for real-time methods C and D. Whereas the price of methods A and B is below one Euro per test, it is around two and eight Euros per test for methods C and D, respectively. Furthermore, in order to perform methods C and D, the laboratory has to be well-equipped with a real-time thermocycler and the corresponding software. This has to be considered for laboratories on a low budget that do not perform real-time PCR assays routinely and therefore do not possess such equipment.

Hands-on and hands-off times in methods A and B are higher compared to real-time methods C and D, due to the fact that they are conventional methods and therefore cycle times are longer (about 2:45 h per run), an agarose gel has to be prepared and electrophoresis has to be conducted for analysis. Furthermore, several reagents have to be ordered separately for these methods. Cycle times of methods C and D are between 1:30 h and 1:45 h. Additionally, results can be obtained directly after the run, which makes methods C and D a lot faster.

In summary, methods A and D showed slight advantages compared to methods B and C. The good performance of method A was not only confirmed by our analysis but has also been shown in external analysis (Schijman et al., 2011; Sturm et al., 1989). It was the only method investigated that targets kDNA, which can be important when examining lineages containing high numbers of minicircles. As a conventional PCR method, it is cheap and no costly real-time PCR equipment is needed. Additionally, it is well-established at the reference center for CD diagnostics in Spain (ISCIII).

Conduction of method D is quick, convenient and easy. It is a commercial kit, which is provided by the company with all reagents. LOD values were very low, showing the high sensitivity of this real-time method. It amplifies Sat-DNA using a highly specific probe, and therefore confirms the presence of *T. cruzi* DNA in the sample. Also,

method D can be used to quantify the parasite load and therefore estimate parasitemia in patients.

4. Conclusions

For routine molecular diagnostics of patients with a positive serological result for CD in a non-endemic region, the following procedure in well-equipped or reference laboratories is recommended by the DITM: Both Methods A and D, targeting kDNA and Sat-DNA, respectively, should be performed. If one of the two tests is positive, the patient has to be considered PCR-positive. Infection with a Discrete Typing Unit (DTU) that has a low number of Sat-DNA, which cannot be detected when targeting Sat-DNA, is one possible explanation for divergent results (positive in method A, negative in method D). Sequence polymorphism due to lineage differences has to be considered. Therefore, it is important to target kDNA and Sat-DNA in two different runs when performing PCR in CD diagnostics.

Furthermore, due to the variability of parasitemia in patients with chronic CD, it might be useful repeating the examination with blood taken at a different points of time. If sufficient funds are available, quantification of the parasite load should be performed using Method D, either with standardized templates provided by the company or a self-made standard curve. Quantification can be an important tool in diagnostics of CD (Melo et al., 2015; Piron et al., 2007) and can be used during and after therapy to control treatment efficacy.

For laboratories which do not have access to real-time PCR thermocyclers, it can be recommended to perform method A only, if molecular diagnostics is needed. For further analysis, the sample should be mailed to a well-equipped reference laboratory in order to perform further tests and, if appropriate, quantification of parasitemia. If pretreatment of samples with guanidine-EDTA is conducted as detailed in Section 2.1 of this article, shipment of samples at ambient temperature is reasonable and affordable.

In order to avoid false positive and false negative results in the future, prospective studies specifically comparing the two targets, kDNA and Sat-DNA, would be useful and of great interest to the medical personnel dealing with chronic chagasic patients.

Conflict of interest

The author Edoardo Marchisio is an employee of Dia.Pro Diagnostic BioProbes srl, S.S.Giovanni, Italy. For all other authors there is no conflict of interest.

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