Title: Molecular diagnostics for Chagas disease – up to date and novel methodologies.

Abstract

Significance: Chagas disease is caused by the parasite *Trypanosoma cruzi*. It affects 7 million people, mainly in Latin America. Diagnosis is usually made serologically, but at some clinical scenarios serology cannot be used. Then, molecular detection is required for early detection of congenital transmission, treatment response follow up, and diagnosis of immune-suppression reactivation. However, present tests are technically demanding and require well-equipped laboratories which make them unfeasible in low-resources endemic regions.

Areas covered: available molecular tools for detection of *T. cruzi* DNA, paying particular attention to quantitative PCR protocols, and to the latest developments of user-friendly molecular diagnostic methodologies.

Expert opinion/Commentary: in the absence of appropriate biomarkers, molecular diagnosis is the only option for the assessment of treatment response. Besides, it is very useful for the early detection of acute infections, like congenital cases. Since current Chagas disease molecular tests are restricted to referential labs, research efforts must focus in the implementation of easy-to-use diagnostic tools in order to overcome the access to diagnosis gap.

Keywords

Chagas disease, *Trypanosoma cruzi*, molecular detection, quantitative PCR, point-of-care, isothermal amplification.

1. Introduction

Chagas disease is a neglected tropical disease (NTD) caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*; order Kinetoplastida; family Trypanosomatidae). WHO estimates there are 7 million people infected worldwide, most of them in Latin America where triatomine vectors (order Hemiptera; family Reduviidae) that transmit the infection are endemic [1]. Several other infection routes have been described, like consumption of parasite contaminated food, from mother to child, through blood transfusion and by organ transplant [1]. The three latter are of relevance also in non-endemic regions (e.g.: Australia, Canada and Europe) where disease has been globalized in the last decades with population flows from endemic regions [2]. In the last report from the WHO, it is estimated that there are ~30,000 new vector-related cases, and ~8,600 new congenital transmission cases per year [3]. Despite its impact in public health there is no available vaccine, yet there are two drugs to treat Chagas disease: benznidazole and nifurtimox [1]. Unfortunately both have severe side effects due to long term dosages and reduced parasitological efficacy in the advanced chronic stage [1, 4]. In contrast, therapy is more effective and less toxic in younger patients, which highlights the paramount importance of an accurate and timed diagnosis [5]. However, Chagas disease remains largely underdiagnosed and as a consequence of its invisibility, chemotherapy barely reaches 1% of the infected people [5].

The featured silence of Chagas disease is rooted to its clinical progression characteristics [2]. Two disease stages can be distinguished and the methodologies to be applied for disease diagnosis are stage-dependent. Firstly, a short acute stage occurs. Parasitemia is patent along it and direct detection can be achieved by parasitological techniques like parasite microscopic observation in blood smears or microhematocrit,

xenodiagnoses, and hemoculture [6]. These methods may involve long culture protocols and often entail poor sensitivities [6]. Bloodstream parasites presence can also be detected by molecular amplification of their genetic material with polymerase chain reaction (PCR) or real-time quantitative PCR (qPCR) methods with higher sensitivity than the aforementioned techniques [6, 7]. However, in a majority of cases acute symptomatology is non-existent or courses as a mild flu and thus the infection mostly goes undiagnosed at this stage.

Surpassed the acute phase, the disease enters in an indeterminate chronic period that may span decades. In ~70% of patients no further clinical symptoms will ever manifest, but in the remaining ~30% severe anomalies will disrupt their heart and/or gastrointestinal tract potentially leading to death if untreated [2]. Throughout the chronic phase, parasite bloodstream presence is intermittent and low, which hampers direct detection. Diagnosis is then made by means of serological assays, like indirect hemagglutination (IHA), indirect immunofluorescence (IIF) or enzyme-linked immunosorbent assays (ELISAs). Accordingly to WHO´s guidelines, at least two serological tests based on distinct antigen sets must agree to establish a conclusive diagnosis due to the wide antigenic variability of the parasite [1]. Nonetheless, the use of a single technique has been recently postulated due to the commercialization of increasingly sensitive and specific tests, like the Architect Chagas (Abbott Laboratories) [8].

As it is in the chronic phase that symptomatology appears, Chagas disease diagnosis is largely made by serological tests. Nevertheless, clinical molecular diagnosis is useful for: (i) improving early detection of congenital transmission in newborns when presence of anti-*T. cruzi* immunoglobulins from the mother can confound serological testing [7, 9]; (ii) follow up of parasite reactivation in immune-suppressed patients, be it *T. cruzi*-HIV co-

infected [10], advanced Chagas cardiomyopathy patients receiving heart transplant [11], or non-infected patients that have received an organ from a *T. cruzi*-positive donor [12, 13]; and (iii) the evaluation of new treatments in clinical trials where serological negative conversion of treated seropositive patients cannot be used because it is impractical from a study time perspective [14, 15]. Besides, molecular diagnosis has also been applied in the pre-clinical setting to assess the anti-*T. cruzi* performance of drugs, like posaconazole, and the proteasome inhibitor GNF6702 [16, 17].

Molecular-based detection of *T. cruzi* is also very important for the study of the parasite eco-epidemiology. More than 100 species of triatomines can spread the infection (e.g. *Triatoma infestans*, *Triatoma dimidiata*, or *Rhodnius prolixus*) [6]. A similar number of susceptible mammalian hosts can get infected (e.g. armadillos, opossums, raccoons, domestic dogs,…) [6], and the vast majority of them lack of specifically designed serological tools. As a result, molecular detection is then required for the understanding of the parasite domestic, peri-domestic and sylvatic biological cycles as well as of their overlapping or non-overlapping nature, which may ultimately translate in a better implementation of vector control programs (insecticide spray of housing and surroundings, dog collars, etc.) [18, 19]. In addition, development of *T. cruzi*-tailored molecular tools has provided major insight on the parasite genetic diversity [20]. This is organized in Discrete Typing Units (DTUs; TcI to TcVI), which have been distinctively associated to disparate ecologies and geographical distributions [21]. Even further diversity has been acknowledged within TcI genotype leading to its subdivision (TcIa-TcIe) [22]. Conclusive studies to address the relation between parasite genotype and disease pathogenesis are to be done, but there is certainly a geographical variation in the prevalence of its cardiac, digestive, and/or cardiodigestive clinical forms, likely related to distinct virulence of the

circulating parasite strains and to the genetic traits of the human populations of each region [12, 23, 24]. In any case, occurrence of co-infections and co-existence of various genotypes in the same patient over time seems to be a common fact [12, 25-28]. This has been mainly looked upon Argentinian and Bolivian patients and the most common combination of genotypes detected was that of II/V/VI [12, 25, 28]. Hence, same as it has been proposed for *T. cruzi* drug discovery programs [29], disease diagnostics must encompass the parasite diversity.

As it happens with Chagas disease conventional serological tests, currently available molecular techniques require of equipped labs and trained personnel for their performance. Such demands are frequently unattainable in low-resources countries. In response, serum or whole blood based immunological rapid diagnostic tests (RDTs) were developed to be implemented in those regions in substitution of conventional serological assays [30]. RDTs are user friendly immune-chromatographic tests amenable to be performed at point of need locations for disease surveillance and diagnosis screening [30]. Likewise, easy-to-use point-of-care (POC) molecular diagnostics would be of great aid for the performance of early diagnosis of congenital transmission and follow up of parasite reactivation in immune-suppressed patients at ill-equipped laboratories. Methods alike isothermal nucleic acids amplifications that do not require thermocyclers nor imaging equipment for results readout [31, 32], or low-cost technological solutions that would substitute expensive and energy demanding current apparatus are being investigated to facilitate accurate molecular diagnosis in low-resources areas [33].

A recent article by a multidisciplinary group of experts conveyed desired Target Product Profiles (TPPs) for the development of Chagas disease diagnostics at three distinct scenarios: (1) POC acute phase diagnosis; (2) POC diagnosis of chronic phase patients; (3)

monitoring of anti-parasitic treatment response [34]. Indeed, establishing TPPs for much-needed diagnostics is a very important first step. Biomarkers, especially for the assessment of drug treatment response, should be considered too; and there are in fact several research groups working on this matter [35]. However, there are still no validated biomarkers in the market for the diagnosis, prognosis and treatment response assessment for Chagas disease [35]. Indeed there is a lot of work to be done. In regards to molecular-based diagnostics, it must begin with the standardization of currently available procedures and the development of POC methodologies amenable to be implemented in low-resources settings.

Structure and methods

A primary aim of the present article is to review recent developments of Chagas disease molecular diagnostics, mainly covered in its first part (section 2). Nonetheless, currently available methodologies, such as qPCR, are unfeasible in many laboratories from endemic regions that should be fitted with molecular tools to diagnose acute and congenital infections. Therefore, this article also conveys late advances in the easy-to-use molecular diagnostics field, which could have a profound impact on Chagas disease control (section 3).

Publications addressing Chagas disease molecular diagnosis were retrieved from PubMed/MEDLINE using the keywords: Chagas disease OR *Trypanosoma cruzi* AND molecular diagnosis OR molecular detection OR polymerase chain reaction. Searches for novel molecular methodologies were made in PubMed/MEDLINE typing neglected tropical diseases OR Chagas disease OR *Trypanosoma cruzi* AND isothermal amplification detection OR loop isothermal amplification. In all cases secondary searches were made

following the first and/or last author´s link as well as PubMed/MEDLINE provided articles indexed in "Similar articles" and "Cited by" sections.

2. Clinical molecular diagnostics - update

2.1. *T. cruzi* DNA detection, efforts to homogenize a very heterogeneous landscape

Many diverse PCR assays have been developed for Chagas disease diagnosis since a first protocol was released in 1989 [36], including conventional PCR, nested PCR, as well as simple and multiplex qPCR [9, 37, 38]. Such diversity has resulted in a heterogeneous set of techniques that often precludes comparison of results between different studies and/or laboratories [39]. The factors that contribute to the variable levels of sensitivity and specificity encountered are: the samples processing, samples preservation conditions, the DNA purification methods used, the distinct *T. cruzi* sequences targeted for amplification, the primers and amplification reagents used, and the thermo-cycling programs followed. With the objective of selecting the best-performing procedures, a multi-site collaborative work led by Alejandro Schijman laboratory (at INGEBI-CONICET, Argentina; co-author of this article) evaluated the performance of up to 48 PCR and qPCR protocols, present in 26 laboratories, over three standard sets of samples (A, B and C; [39]. Sets were conformed so that their analysis would: (A) inform on the limit of detection (LOD) of strains from three distinct lineages (DTUs I, IV and VI); (B) decipher the influence of the DNA extraction method followed; and (C) assess assays´ sensitivity and specificity over well characterized blood clinical samples from distinct origins (Argentina, Bolivia, Paraguay and Brazil) and disease stages (immune-suppressed heart transplanted, indeterminate and chronic symptomatic patients) [39]. Out of this multinational effort four procedures were flagged as best performing, coded LbD/2, LbD/3, LbF/1 and LbQ in the article [39]. All

four targeted multi-copy genes, three of them the nuclear satellite DNA (satDNA) and the remaining one the kinetoplastid minicircle (kDNA). Three relied on solvent DNA extraction while one employed a commercial kit. Two were conventional PCR procedures and the other two qPCR methods (summarized in Table 1). In comparison to serological status of patients, the retrieved sensitivity levels were between 63-74% and all reported 100% specificity [39].

Table 1. PCR and qPCR procedures that have been analytically validated in multicenter international studies.

Coded name*	Extraction method	Target	PCR	Primers		Mastermix	LOD#	LOQ#	Sensitivity†	Specificity†	Refs.
				Names	Sequences						
LbD/2	Solvent	SatDNA	RT	TCZ-F TCZ-R	GCTCTTGCCCACAMGGGTGC CCAAGCAGCGGATAGTTCAGG	Quantitec Sybr-Green (kit)	0.05	ND	69%	100%	[39]
LbD/3	Solvent	SatDNA (182 bp)	C	TCZ-F TCZ-R	GCTCTTGCCCACAMGGGTGC CCAAGCAGCGGATAGTTCAGG	In-House	0.05	N/A	63%	100%	[39]
LbF/1	Roche kit	SatDNA	RT	cruzi1 cruzi2 cruzi3	ASTCGGCTGATCGTTTTCGA AATTCCTCCAAGCAGCGGATA CACACACTGGACACCAA	Roche (kit)	0.46	1.53	63%	100%	[37, 39-41]
LbG/3	Qiagen Dneasy Tissue kit	kDNA	RT	32f 148r 71P	TTTGGGAGGGGCGTTCA ATATTACACCAACCCCAATCGAA CATCTCACCCGTACATT	Applied Biosystems (kit)	0.16	0.90	78%	40%	[41, 42]
LbL/2^	Qiagen DNA blood mini kit	SatDNA (81 bp)	C	Tc-Sat-F Tc-Sat-R	CACTCTCTGTCAATGTCTGTTTGCGTG GAAATTCCTCCAAGCAGCGGATA	OligoC-TesT Coris BioConcept (kit)‡	0.5	N/A	72%	60%	[43, 44]
LbQ	Solvent	kDNA (330 bp)	C	121 122	AAATAATGTACGGGKGAGATGCATGA GGTTCGATTGGGGTTGGTGTAATATA	In-House	0.5	N/A	63%	100%	[39]

*As labeled in reference [39]. #LOD, limit of detection; LOQ, limit of quantification; as determined in [39] for *T. cruzi* CL Brener (TcVI) spiked guanidinium-EDTA blood boiled, except LbF/1 and LbG/3 that were calculated according to the NCCLS guidelines as stated in [41]. †As reported in [39] in comparison to patients serological status. ^The only test that has ever been commercialized for molecular diagnosis of Chagas disease. ‡Sequences for the detection and internal control probes are provided in [44]. The two methods in boldface have been multiplexed with internal amplification controls (IAC) to meet the European Standardization Committee guidelines of standardization for PCR procedures. IAC primer sequences and VIC-TaqMan probes are shown in [40] and [41]. C, conventional PCR; RT, real-time quantitative PCR; ND, not determined; N/A, not applicable. In the RT protocols the third primer corresponds to the probe sequence.

All blood samples were treated with guanidine-HCl 6M-EDTA 0.2 M (pH=8.0) buffer (1:1, buffer:blood; GEB). Samples DNA was extracted from 200 µl of GEB. In regards to the DNA extraction method, despite three of the four best-performers selected relied on solvent extraction [39], the use of commercial kits would be preferred as they favor reproducibility and homogeneity of the procedure in comparison to solvent extraction protocols [39].

In comparison to conventional PCR, qPCR techniques provide a quantitative output in shorter turnaround and are better suited to scale up because they save the gel electrophoresis and gel visualization steps. Despite a slightly more complex equipment is required and each diagnostic determination has a higher cost, the homogeneity, reproducibility and quantitative output make of qPCR the preferred molecular diagnostic to assess treatment response in drug clinical trials [14, 15]. In the absence of reliable biomarkers, consecutive negative results in the detection of *T. cruzi* DNA stands as the surrogate of treatment response in drug clinical trials. However, in the clinical diagnosis

setting, the use qPCR over conventional PCR is liaised to the availability of the required equipment and the cost of real time thermocyclers is much higher than that of conventional ones.

2.2. Real time quantitative PCRs, the preferred option

Amongst the four best performing methods flagged in Schijman et al.[39], the qPCR technique that used a commercial DNA extraction kit [37] has been taken forward for improvement in other studies. Moreira and co-workers coupled it to SYBR green detection instead of its original TaqMan fluorogenic probe [45]. SYBR green has the advantage of being cheaper for amplification of a single target and the cruzi1/cruzi2 primers set dissociation curves indicated high specificity on their own. The study reported equivalent limits of detection (0.4 parasites equivalents per ml) and sensitivity (~70%) as had been shown before [37, 39]. However, if accordingly to the International Standard Organization (ISO) an internal amplification control (IAC) must be carried along for accredited standardization [46], then two test tubes should be arranged per reaction. In contrast, if TaqMan probes are used, the target reaction and its IAC can be multiplexed in a single tube/well easing up the process and saving costs [40]. Duffy and co-workers TaqMan qPCR took Piron et al. procedure a step further by including a previously described IAC derived from a linearized plasmid containing an *Arabidopsis thaliana* sequence that was spiked in the tubes as part of the reaction [38]. The multiplex satDNA qPCR performance was thoroughly analyzed following ISO 16140 guideline [47, 48]. The method showed to be appropriately selective for *T. cruzi* (LOD <1 fg/µl for all DTUs except TcIV), since no *Leishmania* spp. was amplified at all, and cross-reaction with the closely related *Trypanosoma rangeli* occurred only when 10 pg/µl of its DNA was used as template [40].

More recently, the best satDNA and kDNA qPCRs from [39] were IAC upgraded and their performance validated in an international study [41]. The duo of TaqMan multiplexed qPCR methods targeted to kDNA and satDNA and carrying their corresponding *A. thaliana* derived IAC were analyzed on the basis of the parameters of the ISO 16140 guideline [46]. Comparison of both methods in the same lab using the same DNA extraction protocols, amplification reagents, thermocyclers, and quality controls showed that kDNA-based qPCR achieved better sensitivity due to its lower limits of detection and quantification[41]. SatDNA limits of detection of lineages TcI and TcIV were reduced due to their lower satellite gene content [41]. Nonetheless, kDNA-based qPCR still presented the issue of potential cross-reactivity to *T. rangeli* DNA as 10 fg/µl sufficed for its detection. Thus, particular attention to potential false positives must be paid in regions where both parasites prevail if only kDNA method is used (Guatemala, Panamá, Colombia, Venezuela and certain regions of Brazil; [41]).

Clinical sensitivity of both methods in detection of acute cases was 100% (11/11) and all these samples were quantifiable but one by satDNA [41]. The best results of satDNA qPCR described by Duffy et al. had been as well achieved with the detection of acute samples derived from an oral transmission outbreak in Venezuela and due to congenital transmission with, respectively, 87.5% (11/16) and 100% (3/3) sensitivity compared to serology and microhematocrite in each case [40]. In contrast, sensitivity of qPCR methods to diagnose chronic patients, either asymptomatic or symptomatic was below 60% in comparison to serology in [39]. Reported levels of sensitivity by Ramirez et al. were respectively 80.7% (117/145) and 84.1% (122/145) for satDNA and kDNA qPCR methods, which managed to quantify 32.5% and 45.9% of those detected samples [41]. Clinical specificity of qPCR best-performing methods in [39] and multiplexed satDNA and kDNA

qPCRs from [41] is not an issue. It was 100% in all cases as no amplification was achieved from the samples of seronegative controls [39, 41].

2.3. How to circumvent the lack of sensitivity?

Due to the disease characteristics, presence of bloodstream circulating parasites in the chronic stage is scarce [1, 2]. In a prospective study with chronically infected pregnant women attending the Service of Obstetrics in a hospital at Buenos Aires (Argentina), time spaced (at least 4 weeks apart) serial sampling and performance of two to three PCR detections was shown to increase the sensitivity of a kDNA targeted technique [49]. It jumped from 75.6% sensitivity with one sample detection to 95.6% when the output of three serial samples was considered [49]. In the referred study, blood was obtained during pregnancy follow up and up to three qPCR detections were made. In most endemic regions such an approach would be unfeasible in terms of required infrastructure and dedicated costs.

T. cruzi wide genetic diversity and the fact that some lineages are more prevalent in certain regions than in others might involve that the same qPCR procedure performs differently depending on the origin of the specimen [41]. In an attempt to increase the probability of DNA amplification, combination of various qPCR protocols has been proposed in order to overcome poor sensitivities and accuracy issues of using a single determination method [41, 42]. A diagnostic algorithm that included three distinct qPCR techniques was suggested by Qvarnstorm and co-workers [42]. The chosen methods were the best performing satDNA protocol (LbF/1 in [39]; Table 1), the best qPCR of all those targeting kDNA (labeled LbG/3 in [39]; Table 1), and a very specific (though poorly sensitive) protocol targeted to the 18S-rRNA region (coded LbS/3 in [39]; Table 1). As expected, kDNA qPCR showed higher sensitivity. However, cross-reactivity with *T. rangeli* DNA

has been highlighted to potentially impact on the specificity of kDNA targeted qPCR methods due to the homology of the amplified region between this parasite and *T. cruzi* [42]. Although the diagnostic outcome indicated a better performance than using a method alone, the algorithm did not show a major improvement. Furthermore, this kind of diagnostic algorithm based on three qPCRs can only be achieved in well-equipped labs that process a very low number of samples, but realistically it will not be possible to implement in most labs day-to-day dealing with Chagas disease diagnosis.

Very recently, a new qPCR assay has been described to be able detect very low levels of parasite DNA (0.005 fg/µl for TcI strain K98 and 0.01 fg/µl for TcVI strain CL-Brener) [50]. The assay was specifically developed for the assessment of drug treatment in the clinical trial STOP CHAGAS [51] using as sample blood collected with PAXgene tubes [50]. It is based on a previously published kDNA qPCR [42]that has been multiplexed to include the *A. thaliana* IAC [50]. Further modifications have been made to improve its sensitivity, like increasing the proportion of lysis buffer to blood in the specimens processing, redesigning the probe to optimize the sequence and fluorophore, and using new kits for the DNA purification (Quick-gDNA Blood MiniPrep kit, by Zymo Research) and the qPCR amplification Master Mix (1x TaqMan Universal Master Mix II with UNG, by Thermo Fisher Scientific) [50].

2.4 Sample processing and the inclusion of quality controls

The lack of sensitivity and the heterogeneity of retrieved results are respectively related to the infection dynamics in the chronic stage of the disease and to the complex genomic organization and wide genetic diversity of the parasite. All the steps required to go from the patient to a diagnosis outcome must be taken into account to try to overcome the aforementioned hurdles; or at least limit their impact. Before doing the PCR, the molecular

diagnostic path includes blood sampling, blood specimen processing, DNA extraction (usually by a commercial kit with or without modifications), and the amount of DNA used for the amplification.

Blood is obtained by venous puncture in adults and newborns from 1 month of age onwards. Collected volume differs from the averaged 10 ml of the adults to the 1-2 ml obtained from newborns [7, 9, 38]. Anyhow, from the moment blood specimens are collected, start the differences between protocols (Table 2). Some collect the blood in EDTA tubes and store it frozen [37, 42]. Others prefer to mix it 1:1 (blood:buffer) with guanidine-HCl 6M/EDTA 0.2M at pH=8.0 to yield GEB. This can be kept at 4 ºC for months without compromising results [39, 41]. Besides allowing refrigerated storage, GE buffer de-structures the DNA thus facilitating its subsequent amplification. More recently, the use of PAXgene blood collection tubes has also been described [50]. These are easy handling and may provide enhanced workflow efficiency when used with its homonymous blood DNA purification kit. In the procedure developed by Wei et al., PAXgene blood tubes collected specimens were mixed 1:1 with a commercial lysis buffer (GE is made in house) before further purifying the DNA [50]. Dried blood spots in FTA cards have also been used for Chagas disease

Table 2. Variety of blood specimen processing methods and DNA extraction protocols used in T. cruzi DNA quantitative detection algorithms.

[Reference]	Specimen processing	DNA extraction			
		Treated blood vol.	**Commercial kit**	**Kit modifications**	**DNA vol. for PCR**
[37]	EDTA collection tubes and stored frozen	100 µl	High Pure PCR Template Preparation (Roche)	N/A	5 µl
[45]	GEB* - Boiled	200 µl	QIAamp DNA Mini kit (Qiagen)	No proteinase K and elution in 50 µl	2 µl
[40]	GEB - Boiled and not boiled	300 µl§	High Pure PCR Template Preparation (Roche)	N/A	5 µl
[50]	PAXgene tubes + Genomic Lysis Buffer (Zymo Research)†	400 µl‡	Quick-gDNA Blood Mini Prep (Zymo Research)	Elution in 50 µl	2 µl

*GEB states for 1:1 (vol:vol) guanidine-HCl 6M/EDTA 0.2 M (at pH=8.0) mix with blood. GEB samples are stored at 4 ºC. §300 µl of GEB was mixed with 100 µl of the kit´s binding solution and 5 µl IAC, and treated with 40 µl proteinase K. †PAXgene collected blood was mixed 1:1 (vol:vol) with Genomic Lysis Buffer (Zymo Research), and allowed a 10 min lysis step at room temperature (RT) before storage at -80 ºC. ‡400 µl of lysed blood was further mixed with 600 µl of lysis buffer and 5 µl IAC, and let 10 min at RT before DNA extraction.

diagnosis, but blood collection in this format has just been applied to serological detection of *T. cruzi* specific IgGs [52].

Published diagnostic algorithms also vary regarding the volume of treated blood input for the DNA extraction protocol, as well as with the commercial purification kits used (Table 2). Some attach to the kits manufacturers´ instructions, whereas others have introduced slight modifications to them, like Moreira et al. that do not apply proteinase K digestion to their samples and eluate the DNA is half the indicated volume [45]. Noteworthy, an increased sensitivity of satDNA and kDNA qPCRs has been described if DNA is extracted from blood buffy coat, which is rich in nucleated blood cells [42]. It follows the same parasite concentration principle as the parasitological microhematocrite method [53]. Buffy coat is obtained upon centrifugation of the blood specimen at 2,500 g for 10 minutes that segregates the plasma from the cellular blood fraction. The former is removed and the DNA is extracted from the cells [42, 54]. Nonetheless the use of buffy coat as departure point for the DNA extraction has not been generalized because it involves an additional step. The simpler it is the manipulation of the sample, the lesser will be the chances to make a mistake and suffer cross-contaminations leading to false positive results. Something similar occurs with the boiling of the GEB specimens. Despite increased analytical and clinical sensitivities have been observed when using boiled samples [40], such boiling is not advised when testing a big amount of samples (for instance in a clinical trial) as it entails a risk of contamination of the negative samples.

Independently of the heterogeneity of procedures, quality controls must be included to limit the risks of reporting false negative results. Some studies for quantification of *T. cruzi* parasitic loads have included a host DNA sequence, e.g. RNase P human gene, as IAC [37, 41, 45]. Despite this is useful for qualitative purposes, the use of a heterologous

intrinsic IAC such as RNase P should not be recommended. This is because the content of human blood cells can be highly variable between samples as it depends on the nutritional, metabolic, and immunologic status of the patients [40]. The use of a heterologous extrinsic IAC like the linearized pZErO-2 recombinant plasmid with an inserted sequence *A. thaliana* aquaporin is more advisable [38]. Besides serving as IAC, by spiking a normalized amount of the plasmid in the samples before doing the DNA extractions, the whole procedure can be monitored [38, 41, 50]. This kind of heterologous extrinsic IAC is more advisable than homologous extrinsic or heterologous intrinsic controls, as there will not be competition with the target sequence, nor will potentially overabundant host genetic materials shade any inhibitory effects on parasite DNA amplification, plus the variability of host DNA content between samples will be avoided [40].

As it can be observed, present methodologies are complex and expensive. Indeed they are useful for the evaluation of drug treatment response in clinical trials and for the performance of clinical diagnosis in well-equipped referential laboratories in endemic and non-endemic regions. However, their complexity and cost preclude their implementation to service the molecular diagnosis of the disease in vast areas of endemic regions that are low-resourced and endure poor investment.

3. New tools for Chagas disease point-of-care molecular diagnosis

A first attempt to ease up molecular diagnosis of Chagas disease was based on the oligochromatographic OligoC-TesT technology [44]. Although still relying on the above depicted series of sequential events (blood DNA extraction, thermocycler sequence amplification), its strips layout permitted naked eye visualization of results in a quick disposable format rather than by tedious agarose gel imaging or through the more

expensive real time thermocyclers [44]. Initially designed to target *T. cruzi* satellite DNA, a kDNA-based OligoC-TesT was later on described for increased sensitivity [43]. So far, an OligoC-TesT assay has been the only commercially available molecular tool for Chagas disease diagnosis (Coris BioConcept, Gembloux, Belgium; marked with ^ in Table I). All required reagents for PCR amplification as well as running buffers and strips were included in the kit, but its production had to be discontinued due to unfavorable market response (Coris BioConcept Department ClientCare communication). Its dependence on conventional thermal cycling equipment might have been the cause behind OligoC-TesT commercialization failure.

In low-resource settings that lack the infrastructure, equipment and technical skills to support the use of PCR or qPCR as molecular diagnostics, new isothermal molecular technologies would be particularly amenable [32]. Amongst them, loop-isothermal amplification (LAMP; Eiken Co., Japan) and recombinase polymerase assay (RPA; Alere, USA and TwistDx, UK) stand out due to their low performance temperatures and fast turnaround of results.

Loop-mediated isothermal amplification (LAMP) of *T. cruzi* DNA has been researched [55]. LAMP does not require expensive electrically demanding thermocyclers but a simple water bath or heat block device, and results can be naked eye visualized within an hour time. It is based on *Bacillus stearothermophilus* (*Bst*) DNA polymerase large fragment and a set of 4 to 6 primers that allow highly specific, rapid and efficient DNA amplification at an isothermal 65 ºC step [56]. These characteristics make an ideal POC diagnostic methodology of it, and as such it is being developed for a plethora of tropical infectious diseases [31, 57-62]. The technology has been thoroughly studied for the diagnosis of Human African Trypanosomiasis (HAT) and Leishmaniasis, respectively

caused by the *T. cruzi* closely related kinetoplastid parasites *Trypanosoma brucei* (*gambiense* or *rhodensiense*) and *Leishmania* spp [63]. Recently, a LAMP assay with dried reagents stabilized in a single tube with long shelf life capable of specifically amplifying *T. brucei gambiense* and *T. brucei rhodensiense* DNA directly from detergent-lysed blood samples was described [62]. This LAMP detection system has been refined to allow bedside diagnosis and field surveillance by adding to it a portable battery system to power a trans-illuminator for improved performance. In a recent case report, LAMP blood detection of *T. brucei rhodensiense* was shown [64]. Upon larger field studies, this methodology could definitely be a major breakthrough towards HAT control [62]. Several studies are also applying LAMP for Leishmaniasis clinical diagnosis [57, 65, 66]. A recent work with clinical samples from just two patients (blood, saliva and tissue) showed that the method could be used with crude samples uncompromising sensitivity compared to qPCR, as far as samples were boiled previous to LAMP [65]. Samples boiling preparatory step has been described for *Schistosoma haematobium* LAMP assay too [61].

In contrast, LAMP peer reviewed application for Chagas disease diagnosis limits to Thekisoe and co-workers published LAMP method that was developed to discriminate between *T. cruzi* and *T. rangeli* infections in field collected triatomine vector *Rhodnius pallescens* [55]. The designed primers targeted the 18S rRNA and the small nucleolar RNA (snoRNA) genes, respectively of *T. cruzi* and *T. rangeli*. They showed parasite specific DNA amplification respect to human or vector derived DNA, and a sensitivity of 100 fg and 1 pg per reaction respectively for *T. cruzi* and *T. rangeli* DNA [55]. In comparison to the above mentioned qPCR methods, the *T. cruzi*-LAMP sensitivity was >100 fold poorer which may be due to the selected target (18S rRNA gene). Despite it was published in 2010 no further references to the application of LAMP in Chagas disease clinical diagnosis could

be retrieved from PubMed. Nonetheless, a LAMP test for Chagas congenital transmission diagnosis would be a much desired target as due to its characteristics will come to fill a diagnostic gap in congenital Chagas disease transmission [34, 67]. Currently, based on Eiken Co. LAMP technology design, FIND and collaborators, amongst which is Alejandro Schijman laboratory at INGEBI, set up a *T. cruzi* LAMP assay targeted to the highly repetitive satDNA sequence [68]. The amplification reaction takes 40 minutes at 65 ºC and a subsequent 5 more minutes at 80 ºC to inactivate the enzyme. Prototype assay microtubes already contain the required reagents dried in their caps, and for direct naked eye visualization calcein was used. The assay showed very good inclusivity and selectivity as DNA from *T. cruzi* stocks belonging to the six DTUs was detected (Figure 1; [68]). The test sensitivity was analytically assessed in comparison to its counterpart satDNA qPCR on serial dilutions of *T. cruzi* DNA samples, as well as on EDTA and heparinized blood samples that were spiked with known amounts of parasite epimastigotes. In terms of clinical diagnosis, LAMP assay was positive in congenital and immunosuppressed Chagas disease samples, although chronic patients´ samples were only detectable by qPCR (with Ct values below the LOQ). Further details of this assay will (hopefully) be available soon as the article describing them is currently under review (Alejandro Schijman´s communication).

On the other hand, RPA couples isothermal enzymatically-driven primer targeting with strand-displacement DNA synthesis [69]. It provides faster turnaround and works at lower temperature than LAMP [32]. Furthermore, its assay design is also less complex than that of LAMP and the results readout can be linked to lateral flow visualization of the amplification as it has been described for the detection of *Leishmania infantun* DNA in dogs [70]. Samples DNA extraction would still pose a conundrum to surmount for bedside

diagnosis under field conditions. For that, a mobile lab based on RPA that also considers the DNA extraction process has been designed for point of need diagnosis of *Leishmania donovani* human infections [71]. Contained in two suitcases, one for DNA extraction with a fast commercial method (SpeedXtract, Qiagen) and the other for the performance of the amplification reaction, the system can be powered by a portable generator and a solar panel to re-charge it [71]. Other technical solutions could serve as an alternative to isothermal amplification reactions, like recent works developed by Wong and collaborators at AI Biosciences Inc. (College Station, Texas, USA). Amongst the inventions they have devised that would be useful for field molecular diagnosis there is an inexpensive thermocycler (less than $200) built up with thermos, which performance has been shown to match that of commercial thermocyclers at a fraction of their cost [33]. PCR tubes are wire-held from an arm that is coupled to a micro-servo controlled by a programmable microcontroller (Figure 2; extracted from reference [58]). The system is fed with a small portable battery and overall consumes a lot less electricity per run than a classic thermocycler which redounds in a cost save. The invention, named thermos thermal cycler (TTC), was shown to be specific, sensitive and provide results within 30 minutes for target sequences up to 1.5 Kb long [33]. In a subsequent article TTC capability to detect *Chlamydia trachomatis* DNA extracted from positive urine samples was proved [72]. TTC system managed so well with temperature stability that it also permitted performance of RNA detection from human serum samples spiked with Ebola virus, HIV or Dengue virus RNAs, and even outdid the commercial reverse transcription qPCR methods brought along for comparison [72]. Amplification reagents, reactions set up and tubes required are the same as the ones required for traditional thermocyclers-based protocols. TTC could therefore allow molecular detection in low-resource settings with currently available reagents as far as it

gets linked to low-cost detection technologies like nucleic acids lateral flow or smartphone-mediated fluorescence detection [73, 74]. Nonetheless, DNA extraction from the samples would still be an issue. In another article, AI Biosciences Inc. team provides a solution for this in the form of a re-purposed 3D-printer modified for automated DNA extraction [74]. Even the printer´s heated bed heat can be re-directed to heat water baths in order to perform nucleic acids amplification reactions [74].

So far, the clinical samples detected by TTC were urine of people infected with *Chlamydia trachomatis*, which is a bacteria species that causes urogenital infections. Detection of *T. cruzi* DNA in urine of infected individuals is less likely, as it does not damage the kidney or the urinary tract and cell-free circulating DNA fragments that may cross the trans-renal barrier towards being secreted in the urine have been described to be 150-200 bp in size [75]. Indeed, the use of urine or saliva as samples for POC diagnosis was agreed in the Chagas disease TPPs document [34]. In this regards, current efforts undertaken relate to direct detection of parasite antigens in those samples [76, 77].

Expert commentary

Molecular tests should not be ordered for the clinical management of Chagas disease chronic patients [39, 78]. It is not just the parasite genetic diversity that complicates a sensitive detection, but also the inherent limitation imposed by the biological behavior of the disease with low and intermittent parasitemias in its chronic phase. However, molecular detection is the only method currently available to be used as surrogate marker of treatment success or failure in drug studies with serologically positive patients [14, 15]. Furthermore, molecular tests have proved highly sensitive in detection of acute infections, like those occurring by congenital or oral *T. cruzi* transmission, as well as in anticipating disease re-

activation in immune-suppressed patients [6]. More emphasis must be given to its implementation in these particular scenarios and standardized widespread protocols must be made available to the healthcare community. Ideally, the definitive qPCR method should be a single assay rather than a combination of methods to minimize costs. The former said, if the agreed diagnostics TPP guidelines are to be attached to, qPCR techniques, single or multiplexed, would not be desirable at all as they are not simple, nor cheap, cannot be performed at POC site, and require preceding sample preparation steps [34]. In other words, they might be applicable at main reference laboratories and hospitals in large urban areas but difficult to implement in primary health centers with insufficiently equipped labs. Therefore, easy to use molecular diagnostics to be performed at POC sites by trained personnel (not necessarily molecular biology specialists) must be pursued. The participation of the industry in the development of commercial and standardized tests is suitable as taking into account the high and widespread impact of Chagas disease a large demand is expected. Nonetheless, these tests have to be easy to implement, should be evaluated independently by reference laboratories and must be cheaply acquired over the counter.

Field deployment and implementation of POC molecular diagnostics would mean a major breakthrough, especially for congenital transmission control. Although it varies geographically it is estimated that ~5% of newborns to Chagas disease women are infected [79]. Now that blood banks are screened and vector transmission is receding in many regions, congenital route is doomed with ~25% of new infections [49, 79]. Since drug treatment within the first year of age is 90-100% effective, early *T. cruzi* diagnosis of pregnant women and their newborns becomes crucial and must be included in the standard of care in endemic regions or whenever there is any suspicion of *T. cruzi* infection in the

mother [2, 7, 9, 79]. In the case of molecular diagnosis of congenitally transmitted *T. cruzi* in umbilical cord blood samples collected at birth, although no scientific reference has yet proved it, parasite DNA from maternal origin could be detected giving rise to a false positive result. [79]. Therefore, a confirmatory test should be made a month later. By then, an increase in the parasitic load will ease the detection, now made upon peripheral blood extracted from the newborn [7]. In many endemic areas the sampling and testing at birth (generally by micromethod) is performed in an attempt to ensure the attachment of the mothers to the health follow-up protocol. Despite PCR has been shown to allow an earlier diagnosis of congenital infection [7, 9], its use has not been implemented in the health systems of endemic countries due to a lack of resources. Current guidelines for congenital Chagas disease diagnosis rely on parasitological detection by micromethod and serological assessment >8 months after birth [79]. The micromethod has been shown to be less sensitive than PCR [7, 9], and by the time a serological diagnosis is achieved a precious time for treatment has been lost. This is a diagnostic scenario where cost-effective easy-to-use molecular tools, such as LAMP or RPA could play a major role.

Five-year view

In order to make diagnosis (and treatment) available to more people, low-cost point of need diagnostics, amenable in low-resource settings, must be widely implemented in the territory. Importantly, these should involve minimal interventions and ideally work with POC samples, such as a few blood drops or urine [34]. Chagas disease diagnosis should take into account the distinct clinical diagnostic settings and the field conditions found in many Chagas disease endemic regions. Serological screening, meaningful to diagnose chronic patients, could be simplified and widespread by means of RDTs implementation.

Congenital transmission diagnosis and immune-suppressed patients follow-up, that require parasite detection, could be simplified using POC molecular detection methods. This would at the same time crucially reduce the time to treatment increasing chances to positively respond to it, a key feature in congenital cases when treatment efficacy is almost 100%. In order to establish the best diagnostic strategy, population based studies should be performed to show that these alternative methodologies perform at least as good a currently available impractical diagnostics. Several RDTs are now available, and some advances have been made in their field validation (Egüez K et al., PLoS Negl Trop Dis 2017 accepted for publication). In stark contrast, *T. cruzi* easy-to-use molecular diagnostics landscape looks flat. However, given the complexity and costs of current qPCR methods, their arrival is largely expected. What appears clear is that with current tools Chagas disease diagnosis and treatment is barely reaching a fraction of those infected.

Key issues

- Chagas disease affects 8 million people worldwide and there are two drugs available against it: benznidazole and nifurtimox. They have toxic side effects and show diminished efficiency the longer the infection, but are well tolerated by children and have high efficacy in acute and early diagnosed cases.
- The disease is largely underdiagnosed because of a mostly asymptomatic acute stage and a long lasting indeterminate phase which results in barely 1% of infected people receiving treatment. Furthermore, diagnosis often arrives when symptoms are advanced and available drugs are less efficient.

- With currently available serological and molecular-based diagnostics the access to a Chagas disease diagnosis in vast endemic regions is limited. Development and implementation of easy to use point-of-need diagnostics are urgently needed.
- Molecular diagnosis of chronic Chagas disease is not recommended. But molecular diagnostics could be useful for early detection of congenital infection, assessment of infection reactivation in immune-suppressed patients, and for follow-up of drug treatments. In all cases, seropositive status of patients precludes the use of serological diagnostics.
- Standardization of currently available molecular diagnostic procedures is paramount for reliable inter-lab and inter-studies comparisons. Recent international multicenter efforts have been conducted to canalize diversity into a few best performing assays. If not gold-standard is ever available, at least homogenous methodologies should be widespread implanted for clinical studies validation.
- Molecular diagnosis of congenital infection transmission is not yet accepted and widely distributed despite it is faster and more sensitive than classical parasitological methods. A large study is needed to show that a PCR tests may suffice to trigger treatment of newborns that are very reactive to chemotherapy and show extremely good cure rates.
- Towards an affordable field deployment of molecular diagnosis, especially in remote regions, the use of new methodological approaches should be implemented. LAMP, RPA or TTC could be an option. Other contribution could be the hacked 3D printer developed by AI Biosciences Inc. that solves the upfront sample processing and allows amplification in the same low-cost apparatus.

- Rapid, low-cost diagnostics and a reliable access to treatments are instrumental towards the control of Chagas disease, and will definitely result in a health status improvement of large segments of the population in Latin America.

References

1. Chagas disease (american trypanosomiasis) fact sheet. Centre WHOM, 2016. Available at: http://www.who.int/mediacentre/factsheets/fs340/en/ [Last accessed]
2. Pinazo MJ, Gascon J. Chagas disease: From latin america to the world. Reports in Parasitology 2015; 4:7-14
3. Organization WH. Chagas disease in latin america: An epidemiological update based on 2010 estimates. 2015 6 90 33-44 WHO Weekly epidemiological record Genève (Switzerland).
4. Morillo CA, Marin-Neto JA, Avezum A, et al. Randomized trial of benznidazole for chronic chagas' cardiomyopathy. N Engl J Med 2015; 373:1295-306
5. Pecoul B, Batista C, Stobbaerts E, et al. The benefit trial: Where do we go from here? PLoS Negl Trop Dis 2016; 10:e0004343
6. Bern C, Kjos S, Yabsley MJ, Montgomery SP. Trypanosoma cruzi and chagas' disease in the united states. Clin Microbiol Rev 2011; 24:655-81
7. Bua J, Volta BJ, Perrone AE, et al. 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

8. Abras A, Gallego M, Llovet T, et al. Serological diagnosis of chronic chagas disease: Is it time for a change? J Clin Microbiol 2016; 54:1566-72

9. Velazquez EB, Rivero R, De Rissio AM, et al. Predictive role of polymerase chain reaction in the early diagnosis of congenital trypanosoma cruzi infection. Acta Trop 2014; 137:195-200

10. Burgos JM, Begher SB, Freitas JM, et al. Molecular diagnosis and typing of trypanosoma cruzi populations and lineages in cerebral chagas disease in a patient with aids. Am J Trop Med Hyg 2005; 73:1016-8

11. Diez M, Favaloro L, Bertolotti A, et al. Usefulness of pcr strategies for early diagnosis of chagas' disease reactivation and treatment follow-up in heart transplantation. Am J Transplant 2007; 7:1633-40

12. Burgos JM, Diez M, Vigliano C, et al. Molecular identification of trypanosoma cruzi discrete typing units in end-stage chronic chagas heart disease and reactivation after heart transplantation. Clin Infect Dis 2010; 51:485-95

13. Cura CI, Lattes R, Nagel C, et al. Early molecular diagnosis of acute chagas disease after transplantation with organs from trypanosoma cruzi-infected donors. Am J Transplant 2013; 13:3253-61

14. Molina I, Gomez i Prat J, Salvador F, et al. Randomized trial of posaconazole and benznidazole for chronic chagas' disease. N Engl J Med 2014; 370:1899-908

15. Alvarez MG, Hernandez Y, Bertocchi G, et al. 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 2016; 60:833-7

16. Khare S, Liu X, Stinson M, et al. Antitrypanosomal treatment with benznidazole is superior to posaconazole regimens in mouse models of chagas disease. Antimicrob Agents Chemother 2015; 59:6385-94

17. Khare S, Nagle AS, Biggart A, et al. Proteasome inhibition for treatment of leishmaniasis, chagas disease and sleeping sickness. Nature 2016; 537:229-33

18. Sosa-Estani S, Segura EL. Integrated control of chagas disease for its elimination as public health problem--a review. Mem Inst Oswaldo Cruz 2015; 110:289-98

19. Tarleton RL, Reithinger R, Urbina JA, et al. The challenges of chagas disease--grim outlook or glimmer of hope. PLoS Med 2007; 4:e332

20. Messenger LA, Yeo M, Lewis MD, et al. Molecular genotyping of trypanosoma cruzi for lineage assignment and population genetics. Methods Mol Biol 2015; 1201:297-337

21. Zingales B, Miles MA, Campbell DA, et al. The revised trypanosoma cruzi subspecific nomenclature: Rationale, epidemiological relevance and research applications. Infect Genet Evol 2012; 12:240-53

22. Guhl F, Ramirez JD. Trypanosoma cruzi i diversity: Towards the need of genetic subdivision? Acta Trop 2011; 119:1-4

23. Messenger LA, Miles MA, Bern C. Between a bug and a hard place: Trypanosoma cruzi genetic diversity and the clinical outcomes of chagas disease. Expert Rev Anti Infect Ther 2015; 13:995-1029

24. Macedo AM, Segatto M, Implications of *trypanosoma cruzi* intraspecific diversity in the pathogenesis of chagas disease. , in American trypanosomiasis - chagas

disease one hundred years of research., J Telleria, and M Tibayrenc, Editors. 2010, Elsevier: Amsterdam.

25. Martinez-Perez A, Poveda C, Ramirez JD, et al. Prevalence of trypanosoma cruzi's discrete typing units in a cohort of latin american migrants in spain. Acta Trop 2016; 157:145-50

26. Bontempi IA, Bizai ML, Ortiz S, et al. Simple methodology to directly genotype trypanosoma cruzi discrete typing units in single and mixed infections from human blood samples. Infect Genet Evol 2016; 43:123-9

27. Abras A, Gallego M, Munoz C, et al. Identification of trypanosoma cruzi discrete typing units (dtus) in latin-american migrants in barcelona (spain). Parasitol Int 2016; 66:83-88

28. Abras A, Gallego M, Munoz C, et al. Identification of trypanosoma cruzi discrete typing units (dtus) in latin-american migrants in barcelona (spain). Parasitol Int 2017; 66:83-88

29. Zingales B, Miles MA, Moraes CB, et al. Drug discovery for chagas disease should consider trypanosoma cruzi strain diversity. Mem Inst Oswaldo Cruz 2014; 109:828-33

30. Sanchez-Camargo CL, Albajar-Vinas P, Wilkins PP, et al. Comparative evaluation of 11 commercialized rapid diagnostic tests for detecting trypanosoma cruzi antibodies in serum banks in areas of endemicity and nonendemicity. J Clin Microbiol 2014; 52:2506-12

31. Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (lamp): Recent progress in research and development. J Infect Chemother 2013; 19:404-11

32. James A, Macdonald J. Recombinase polymerase amplification: Emergence as a critical molecular technology for rapid, low-resource diagnostics. Expert Rev Mol Diagn 2015; 15:1475-89
33. Wong G, Wong I, Chan K, et al. A rapid and low-cost pcr thermal cycler for low resource settings. PLoS One 2015; 10:e0131701
34. Porras AI, Yadon ZE, Altcheh J, et al. Target product profile (tpp) for chagas disease point-of-care diagnosis and assessment of response to treatment. PLoS Negl Trop Dis 2015; 9:e0003697
35. Pinazo MJ, Thomas MC, Bua J, et al. Biological markers for evaluating therapeutic efficacy in chagas disease, a systematic review. Expert Rev Anti Infect Ther 2014; 12:479-96
36. Moser DR, Kirchhoff LV, Donelson JE. Detection of trypanosoma cruzi by DNA amplification using the polymerase chain reaction. J Clin Microbiol 1989; 27:1477-82
37. Piron M, Fisa R, Casamitjana N, et al. Development of a real-time pcr assay for trypanosoma cruzi detection in blood samples. Acta Trop 2007; 103:195-200
38. Duffy T, Bisio M, Altcheh J, et al. Accurate real-time pcr strategy for monitoring bloodstream parasitic loads in chagas disease patients. PLoS Negl Trop Dis 2009; 3:e419
39. Schijman AG, Bisio M, Orellana L, 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

40. Duffy T, Cura CI, Ramirez JC, et al. 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
41. Ramirez JC, Cura CI, da Cruz Moreira O, 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; 17:605-15
42. Qvarnstrom Y, Schijman AG, Veron V, et al. 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
43. De Winne K, Buscher P, Luquetti AO, et al. The trypanosoma cruzi satellite DNA oligoc-test and trypanosoma cruzi kinetoplast DNA oligoc-test for diagnosis of chagas disease: A multi-cohort comparative evaluation study. PLoS Negl Trop Dis 2014; 8:e2633
44. Deborggraeve S, Coronado X, Solari A, et al. T. Cruzi oligoc-test: A simplified and standardized polymerase chain reaction format for diagnosis of chagas disease. PLoS Negl Trop Dis 2009; 3:e450
45. Moreira OC, Ramirez JD, Velazquez E, et al. Towards the establishment of a consensus real-time qpcr to monitor trypanosoma cruzi parasitemia in patients with chronic chagas disease cardiomyopathy: A substudy from the benefit trial. Acta Trop 2013; 125:23-31
46. (CLSI) CaLSI. Ep17-a2 evaluation of detection capability for clinical laboratroy measurement procedures; approved guideline Edition ed. 2012. Clinical and Laboratory Standards Institute (CLSI). Wayne, Pennsylvania (USA).
47. Anonymous (2002) Microbiology of food and animal feeding stuffs. Protocol for

the validation of alternative methods (en iso 16140). Paris, France: ECf Standardization.

48. NCCLS (2004) Protocols for determination of limits of detection and limits of quantification: Approved guideline. Wayne, Pennsylvania: NCCLS.

49. Bisio M, Seidenstein ME, Burgos JM, et al. Urbanization of congenital transmission of trypanosoma cruzi: Prospective polymerase chain reaction study in pregnancy. Trans R Soc Trop Med Hyg 2011; 105:543-9

50. Wei B, Chen L, Kibukawa M, et al. Development of a pcr assay to detect low level trypanosoma cruzi in blood specimens collected with paxgene blood DNA tubes for clinical trials treating chagas disease. PLoS Negl Trop Dis 2016; 10:e0005146

51. Morillo CA, Waskin H, Sosa-Estani S, et al. Benznidazole and posaconazole in eliminating parasites in asymptomatic t. Cruzi carriers: The stop-chagas trial. J Am Coll Cardiol 2017; 69:939-47

52. Holguin A, Norman F, Martin L, et al. Dried blood as an alternative to plasma or serum for trypanosoma cruzi igg detection in screening programs. Clin Vaccine Immunol 2013; 20:1197-202

53. De Rissio AM, Riarte AR, Garcia MM, et al. 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; 82:838-45

54. Marquez ME, Concepcion JL, Gonzalez-Marcano E, Mondolfi AP. Detection of trypanosoma cruzi by polymerase chain reaction. Methods Mol Biol 2016; 1392:125-41

55. Thekisoe OM, Rodriguez CV, Rivas F, et al. Detection of trypanosoma cruzi and t. Rangeli infections from rhodnius pallescens bugs by loop-mediated isothermal amplification (lamp). Am J Trop Med Hyg 2010; 82:855-60
56. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000; 28:E63
57. Abbasi I, Kirstein O, Hailu A, Warburg A. Optimization of loop-mediated isothermal amplification (lamp) assays for the detection of leishmania DNA in human blood samples. Acta Trop 2016; 162:20-26
58. Beissner M, Phillips RO, Battke F, et al. Loop-mediated isothermal amplification for laboratory confirmation of buruli ulcer disease-towards a point-of-care test. PLoS Negl Trop Dis 2015; 9:e0004219
59. Lucchi NW, Gaye M, Diallo MA, et al. Evaluation of the illumigene malaria lamp: A robust molecular diagnostic tool for malaria parasites. Sci Rep 2016; 6:36808
60. Watts MR, James G, Sultana Y, et al. A loop-mediated isothermal amplification (lamp) assay for strongyloides stercoralis in stool that uses a visual detection method with syto-82 fluorescent dye. Am J Trop Med Hyg 2014; 90:306-11
61. Gandasegui J, Fernández-Soto P, Carranza-Rodríguez C, et al. The rapid-heat lampellet method: A potential diagnostic method for human urogenital schistosomiasis. PLoS Negl Trop Dis 2015; 9:e0003963
62. Hayashida K, Kajino K, Hachaambwa L, et al. Direct blood dry lamp: A rapid, stable, and easy diagnostic tool for human african trypanosomiasis. PLoS Negl Trop Dis 2015; 9:e0003578
63. Stuart K, Brun R, Croft S, et al. Kinetoplastids: Related protozoan pathogens, different diseases. J Clin Invest 2008; 118:1301-10

64. Squarre D, Kabongo I, Munyeme M, et al. Human african trypanosomiasis in the kafue national park, zambia. PLoS Negl Trop Dis 2016; 10:e0004567

65. Sriworarat C, Phumee A, Mungthin M, et al. Development of loop-mediated isothermal amplification (lamp) for simple detection of leishmania infection. Parasit Vectors 2015; 8:591

66. Ghasemian M, Gharavi MJ, Akhlaghi L, et al. Development and assessment of loop-mediated isothermal amplification (lamp) assay for the diagnosis of human visceral leishmaniasis in iran. Iran J Parasitol 2014; 9:50-59

67. Find and eiken chemical co., ltd. Expand collaboration to chagas disease. FIND, 2014. Available at: http://www.finddx.org/news/find-and-eiken-chemical-co-ltd-expand-collaboration-to-chagas-disease/ [Last accessed]

68. Besuschio S, Llano M, Benatar AF, et al. A loop-mediated amplification (lamp) kit for molecular detection of trypanosoma cruzi DNA: A feasibility study. In ASTMH 65th Annual Meeting, 2016; Atlanta, Georgia, USA. Supplement to The American Journal of Tropical Medicine and Hygiene.

69. Piepenburg O, Williams CH, Stemple DL, Armes NA. DNA detection using recombination proteins. PLoS Biol 2006; 4:e204

70. Castellanos-Gonzalez A, Saldarriaga OA, Tartaglino L, et al. A novel molecular test to diagnose canine visceral leishmaniasis at the point of care. Am J Trop Med Hyg 2015; 93:970-5

71. Mondal D, Ghosh P, Khan MA, et al. Mobile suitcase laboratory for rapid detection of leishmania donovani using recombinase polymerase amplification assay. Parasit Vectors 2016; 9:281

72. Chan K, Wong PY, Yu P, et al. A rapid and low-cost pcr thermal cycler for infectious disease diagnostics. PLoS One 2016; 11:e0149150
73. Walker FM, Ahmad KM, Eisenstein M, Soh HT. Transformation of personal computers and mobile phones into genetic diagnostic systems. Anal Chem 2014; 86:9236-41
74. Chan K, Coen M, Hardick J, et al. Low-cost 3d printers enable high-quality and automated sample preparation and molecular detection. PLoS One 2016; 11:e0158502
75. Castro-Sesquen YE, Gilman RH, Yauri V, et al. Detection of soluble antigen and DNA of trypanosoma cruzi in urine is independent of renal injury in the guinea pig model. PLoS One 2013; 8:e58480
76. Castro-Sesquen YE, Gilman RH, Galdos-Cardenas G, et al. Use of a novel chagas urine nanoparticle test (chunap) for diagnosis of congenital chagas disease. PLoS Negl Trop Dis 2014; 8:e3211
77. Castro-Sesquen YE, Gilman RH, Mejia C, et al. Use of a chagas urine nanoparticle test (chunap) to correlate with parasitemia levels in t. Cruzi/hiv co-infected patients. PLoS Negl Trop Dis 2016; 10:e0004407
78. Brasil PE, Castro R, Castro L. Commercial enzyme-linked immunosorbent assay versuspolymerase chain reaction for the diagnosis of chronic chagas disease: A systematic review and meta-analysis. Mem Inst Oswaldo Cruz 2016; 111:1-19
79. Carlier Y, Sosa-Estani S, Luquetti AO, Buekens P. Congenital chagas disease: An update. Mem Inst Oswaldo Cruz 2015; 110:363-8

Figures legends.

Figure 1. *T. cruzi* loop-mediated isothermal amplification (LAMP) assay. A, three views of the prototype microtubes that contain the required reagents dried inside their caps; B, detection of amplified products directly with the naked eye or using a fluorimeter; C, assay detected DNA from *T. cruzi* stocks representative of DTUs I to VI (TcI to TcVI in the figure). +, indicates positive samples; –, indicates negative control tubes (no parasite DNA). *T. cruzi*-LAMP kit is a prototype by Eiken Chemical Co.

Figure 2. Thermos thermal cycler (TTC) setup with three thermos for amplification rounds that require three distinct temperatures. Components shown in the picture include three thermoses, a pan-and-tilt servo to motion the PCR tubes between them, the Arduino electronic controller, a breadboard, and a battery pack. In order to reduce costs, the pan-and-tilt setup is constructed using a soup can and a wood stick, and the PCR tubes holder is made with metal wire. Figure reproduced from reference [72].