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REVIEW

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

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ABSTRACT

Introduction: 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 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.

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1. Introduction

Chagas disease is a neglected tropical disease caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*; order Kinetoplastida; family Trypanosomatidae). The WHO estimates that 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 routes are of relevance also in non-endemic regions (e.g. Europe, Canada, Australia, and Japan) 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 ~8600 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 parasite presence can also be detected by molecular amplification of their genetic material by 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 nonexistent 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, indirect immunofluorescence, or enzyme-linked immunosorbent assays. According to the WHO's guidelines, at least two serological tests based on distinct antigen sets must agree to establish a conclusive diagnosis due to

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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, molecular diagnosis is useful for (1) improving early detection of congenital transmission in newborns when presence of anti-*T. cruzi* immunoglobulins from the mother can confound serological testing [7,9]; (2) follow-up of parasite reactivation in immunosuppressed patients, be it *T. cruzi*-HIV coinfected [10], advanced Chagas cardiomyopathy patients receiving heart transplant [11], or noninfected patients that have received an organ from a *T. cruzi*-positive donor [12,13]; and (3) 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 detection has also been applied in the preclinical 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, and domestic dogs) [6], and for the vast majority of them there are no of specifically designed serological tools available. 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 nonoverlapping nature, which may ultimately translate in a better implementation of vector control programs (insecticide spray of housing and surroundings and dog collars) [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 coinfections and coexistence of various genotypes in the same patient over time seems to be a common fact [12,25–27]. This has been mainly looked upon Argentinian and Bolivian patients, and the most common combination of *T. cruzi* genotypes detected was that of TcII/V/VI [12,25,27]. Hence, same as it has been proposed for *T. cruzi* drug discovery programs [28], 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-resource 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 [29]. RDTs are user-friendly immune-chromatographic tests amenable to be performed at point-of-need locations for disease surveillance and diagnosis screening [29]. 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 immunosuppressed patients at ill-equipped laboratories. Methodologies based on isothermal amplification of nucleicacids amplifications that do not require thermocyclers or imaging equipment for results readout [30,31], or low-cost technological solutions to substitute expensive and energy-demanding current apparatus are being investigated in order to facilitate accurate molecular diagnosis in low-resource areas [32].

A recent article by a group of experts conveyed the 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; and (3) monitoring of antiparasitic treatment response [33]. 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 [34]. However, there are still no validated biomarkers in the market for the diagnosis, prognosis, and treatment response assessment for Chagas disease [34]. Indeed, there is a lot of work to be done. In regard 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-resource settings.

1.1. 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, the article also covers late advancements in easy-to-use molecular diagnostics, 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 following 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 [35], including conventional PCR, nested PCR, as well as simple and multiplex qPCR [9,36,37]. Such diversity has resulted in a heterogeneous set of techniques that often precludes comparison of results between different studies and/or laboratories [38]. The factors that contribute to the variable levels of sensitivity and specificity encountered are the sample processing, sample preservation conditions, the DNA purification methods used, the distinct *T. cruzi* sequences targeted for amplification, the primers and amplification reagents used, and the thermocycling programs followed. With the objective of selecting the best-performing procedures, a multisite collaborative work led by Alejandro Schijman laboratory (at INGEBI-CONICET, Argentina; coauthor 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) [38]. 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 assay sensitivity and specificity over well-characterized blood clinical samples from distinct origins (Argentina, Bolivia, Paraguay, and Brazil) and disease stages (immunosuppressed heart transplanted and indeterminate and chronic symptomatic patients) [38]. Out of this multinational effort, four procedures were flagged as best performing, coded as LbD/2, LbD/3, LbF/1, and LbQ in the article [38]. 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% and 74%, and all four methods reported 100% specificity [38].

All blood samples were treated with guanidine-HCl 6M–EDTA 0.2 M (pH = 8.0) buffer 1:1 (buffer:blood) to yield GEB. DNA was extracted from 200 µl of GEB. In regard to the DNA extraction method, despite three of the four best performers selected relied on solvent extraction [38], the use of commercial kits would be preferred as they favor reproducibility and homogeneity of the procedure in comparison to solvent extraction protocols [38].

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, in particular 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 [14,15]. In the clinical diagnosis setting, the use of 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 qPCRs, the preferred option

Among the four best-performing methods flagged in Schijman et al. [38], the qPCR technique that used a commercial DNA extraction kit [36] was taken forward for improvement in other studies. Moreira and coworkers coupled it to SYBR green detection instead of its original TaqMan fluorogenic probe [44]. SYBR green has the advantage of being cheaper for amplification of a single target, and the cruzi1/cruzi2 primer set dissociation curves indicated high specificity on their own. The study reported equivalent LODs (0.4 parasites equivalents per ml) and sensitivity (~70%) as had been shown before [36,38]. However, if accordingly to the International Standard Organization (ISO), an internal amplification control (IAC) must be carried along for accredited standardization [45], 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 [37].

Duffy and coworkers took Piron et al. TaqMan qPCR [36] a step further by including a previously described IAC [39]. This IAC is a linearized plasmid containing an *Arabidopsis thaliana* sequence that was spiked in the tubes as part of the reaction [37,39]. The multiplex satDNA qPCR performance was thoroughly analyzed following ISO 16140 guideline [46,47]. 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 [39].

Recently, the best satDNA and kDNA qPCRs from [38] were IAC upgraded, and their performance validated in an international study [40]. 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 [45]. 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 LODs and quantification [40]. SatDNA LODs of lineages TcI and TcIV were reduced due to their lower satellite gene content [40]. Nonetheless, kDNA 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, Panama, Colombia, Venezuela, and certain regions of Brazil [40]).

Clinical sensitivity of both methods in detection of acute cases was 100% (11/11), and all these samples were quantifiable but one by satDNA [40]. The best results of the satDNA qPCR described by Duffy et al. had been as well achieved with the detection of acute samples derived from an oral

Table 1. Polymerase chain reaction (PCR) and quantitative PCR (qPCR) procedures that have been analytically validated in multicenter international studies.

Coded name[a]	Extraction method	Target	PCR	Primers		Mastermix	LOD[b]	LOQ[b]	Sensitivity[c] (%)	Specificity[c] (%)	Refs.
				Names	Sequences						
LbD/2	Solvent	SatDNA	RT	TCZ-F	GCTCTTGCCCACAMGGGTGC	Quantitec	0.05	ND	69	100	[38]
				TCZ-R	CCAAGCAGCGGATAGTTCAGG	Sybr-Green (kit)					
LbD/3	Solvent	SatDNA (182 bp)	C	TCZ-F	GCTCTTGCCCACAMGGGTGC	In-House	0.05	N/A	63	100	[38]
				TCZ-R	CCAAGCAGCGGATAGTTCAGG						
LbF/1	Roche kit	SatDNA	RT	cruzi1	ASTCGGCTGATCGTTTTCGA	Roche (kit)	0.46	1.53	63	100	[36,38–40]
				cruzi2	AATTCCTCCAAGCAGCGGATA						
				cruzi3	CACACACTGGACACCAA						
LbG/3	Qiagen Dneasy Tissue kit	kDNA	RT	32f	TTTGGGAGGGGCGTTCA	Applied Biosystems (kit)	0.16	0.90	78	40	[40,41]
				148r	ATATTACACCAACCCCAATCGAA						
				71P	CATCTCACCCGTACATT						
LbL/2[d]	Qiagen DNA blood mini kit	SatDNA (81 bp)	C	Tc-Sat-F	CACTCTCTGTCAATGTCTGTTTGCGTG	OligoC-TesT Coris BioConcept (kit)[e]	0.5	N/A	72	60	[42,43]
				Tc-Sat-R	GAAATTCCTCCAAGCAGCGGATA						
LbQ	Solvent	kDNA (330 bp)	C	121	AAATAATGTACGGGKGAGATGCATGA	In-house	0.5	N/A	63	100	[38]
				122	GGTTCGATTGGGGTTGGTGTAATATA						

[a]As labeled in [38].
[b]LOD is limit of detection and LOQ is limit of quantification, as determined in [38] for *T. cruzi* CL Brener (TcVI)-spiked guanidine HCl-EDTA blood boiled, except LbF/1 and LbG/3 that were calculated according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines as stated in [40].
[c]As reported in [38] in comparison to patient serological status.
[d]The only test that has ever been commercialized for molecular diagnosis of Chagas disease.
[e]Sequences for the detection and internal control probes are provided in [43].
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 [39] and [40]. In the RT protocols, the third primer corresponds to the probe sequence
C: conventional PCR; RT: real-time qPCR; ND: not determined; N/A: not applicable.

transmission outbreak in Venezuela and due to congenital transmission with, respectively, 87.5% (11/16) and 100% (3/3) sensitivity compared to serology and microhematocrit in each case [39]. In contrast, sensitivity of qPCR methods to diagnose chronic patients, either asymptomatic or symptomatic, had been shown to be below 60% in comparison to serological assays [38]. Reported levels of sensitivity for chronic-stage diagnosis 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 [40]. Clinical specificity was not an issue for the best-performing qPCR methods in [38] and the multiplexed satDNA and kDNA qPCRs from [40]. It was 100% in all cases as no amplification was achieved from seronegative samples [38,40].

2.3. How to circumvent the lack of sensitivity (for the chronic stage)?

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 [48]. It jumped from 75.6% sensitivity with one sample detection to 95.6% when the output of three serial samples was considered [48]. 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.

Another feature to consider is *T. cruzi* wide genetic diversity and the fact that some lineages are more prevalent in certain regions than in others [12,23,24]. This might involve that the same qPCR procedure performs differently depending on the origin of the specimen [40]. In an attempt to increase the probability of DNA amplification, the combination of various qPCR protocols has been proposed to overcome poor sensitivities and accuracy issues of using a single determination method [40,41]. A diagnostic algorithm that included three distinct qPCR techniques was suggested by Qvarnstrom and coworkers [41]. The chosen methods were the best-performing satDNA protocol (LbF/1 in [38]; Table 1), the best qPCR of all those targeting kDNA (labeled LbG/3 in [38]; Table 1), and a very specific (though poorly sensitive) protocol targeted to the 18S-rRNA region (coded LbS/3 in [38]). 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* [41]. Although the diagnostic outcome indicated a better performance than using a single method alone, the algorithm did not remarkably improve a single use of the methodologies. 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 it is unfeasible in most labs dealing with Chagas disease diagnosis.

Very recently, a new qPCR assay able to 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) has been described [49]. The assay was specifically developed for the assessment of drug treatment in the clinical trial STOP CHAGAS [50] using as sample blood collected with PAXgene tubes [49]. It is based on a previously published kDNA qPCR [41] that has been multiplexed to include the *A. thaliana* IAC [49]. Several modifications have been made to the original kDNA algorithm in order to improve its sensitivity, such as increasing the proportion of lysis buffer to blood in the specimens processing, redesigning the TaqMan probe to optimize its sequence and fluorophore, and using new kits for the DNA purification (Quick-gDNA Blood MiniPrep kit, by Zymo Research) and qPCR amplification (1x TaqMan Universal Master Mix II with UNG, by Thermo Fisher Scientific) [49].

2.4. Sample processing and the inclusion of quality controls

All the steps required to go from the patient to a diagnosis outcome must be taken into account to try to achieve the best possible performance of the procedure. Beforehand the PCR, the molecular diagnostic path includes blood sampling, blood specimen processing, and DNA extraction (usually by a commercial kit with or without modifications).

For Chagas disease diagnosis, 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 to 2 ml obtained from newborns [7,9,37]. 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 [36,41]. Others mix it 1:1 (blood:buffer) with guanidine-HCl 6 M/EDTA 0.2 M (pH = 8.0) to yield GEB, which can be kept at 4ºC for months without compromising results [38,40]. Besides allowing refrigerated storage, guanidine-HCl/EDTA (GE) buffer de-structures the DNA, facilitating its subsequent amplification. More recently, the use of PAXgene blood collection tubes has also been described [49]. 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 tube-collected specimens were mixed 1:1 with a commercial lysis buffer (GE is made in house) before further purifying the DNA [49]. Dried blood spots in Flinders Technology Associates (FTA) filter paper-based cards have also been used for Chagas disease diagnosis, but blood collection in this format has just been applied to serological detection of *T. cruzi*-specific immunoglobulins [51].

Diagnostic algorithms also differ in the volume of treated blood used for the DNA extraction, as well as in relation to the commercial purification kits used for it (Table 2). Furthermore, some attach to the manufacturers' instructions, whereas others have introduced slight modifications to them, like Moreira et al. that do not apply the proteinase K digestion step and eluate the DNA in half of the kit´s instructed volume [44]. 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 [41]. It follows

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

		DNA extraction			
Ref.	Specimen processing	Treated blood vol. (µl)	Commercial kit	Kit modifications	DNA vol. for PCR (µl)
[36]	EDTA collection tubes and stored frozen	100	High Pure PCR Template Preparation (Roche)	N/A	5
[44]	GEB[a] – boiled	200	QIAamp DNA Mini kit (Qiagen)	No proteinase K and elution in 50 µl	2
[39]	GEB – boiled and not boiled	300[b]	High Pure PCR Template Preparation (Roche)	N/A	5
[49]	PAXgene tubes + Genomic Lysis Buffer (Zymo Research)[c]	400[d]	Quick-gDNA Blood Mini Prep (Zymo Research)	Elution in 50 µl	2

[a]GEB states for 1:1 (vol:vol) guanidine-HCl 6 M/EDTA 0.2 M (at pH = 8.0) mix with blood. GEB samples are stored at 4ºC.
[b]300 µl of GEB were mixed with 100 µl of the kit binding solution and 5 µl IAC and treated with 40 µl proteinase K.
[c]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 −80ºC.
[d]400 µl of lysed blood were further mixed with 600 µl of lysis buffer and 5 µl IAC and let 10 min at RT before DNA extraction.
PCR: polymerase chain reaction; N/A: not applicable; IAC: internal amplification control.

the same parasite concentration principle as the parasitological microhematocrit method [52]. Buffy coat is obtained upon centrifugation of the blood specimen at 2500*g* for 10 min which segregates the plasma from the cellular blood fraction. The former is removed and the DNA is extracted from the cells [41,53]. Nonetheless, the use of buffy coat as sample 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 [39], such boiling is not advised, particularly when testing a big amount of samples like in a clinical trial, as it entails a risk of contamination of the negative samples.

Independently of the featured steps in each procedure, 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 [36,40,44]. 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 [39]. The use of a heterologous extrinsic IAC like the linearized pZErO-2 recombinant plasmid with an inserted sequence of *A. thaliana* aquaporin is preferred [37]. 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 [37,40,49]. In comparison to homologous extrinsic or heterologous intrinsic controls, with a heterologous extrinsic IAC, 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 [39].

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 investments.

3. New tools for Chagas disease POC molecular diagnosis

A first attempt to ease up molecular diagnosis of Chagas disease was based on the oligochromatographic OligoC-TesT technology [43]. Although still relying on the above depicted series of sequential events (blood DNA extraction and 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 [43]. Initially designed to target *T. cruzi* satellite DNA, a kDNA-based OligoC-TesT was later on described for increased sensitivity [42]. So far, an OligoC-TesT assay has been the only commercially available molecular tool for Chagas disease diagnosis (Coris BioConcept, Gembloux, Belgium; marked with [d] in Table 1). 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 [31]. Among them, loop-mediated 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 [31].

LAMP of *T. cruzi* DNA has been researched [54]. LAMP does not require electrically demanding expensive thermocyclers but a simple water bath or heat block device, and results can be visualized by naked eye within an hour time. It is based on *Bacillus stearothermophilus* (*Bst*) DNA polymerase large fragment and a set of four to six primers that allow

highly specific, rapid, and efficient DNA amplification at an isothermal 65ºC step [55]. These characteristics make an ideal POC diagnostic methodology of it, and as such it is being developed for a plethora of tropical infectious diseases [30,56–61]. The technology has been thoroughly studied for the diagnosis of human African trypanosomiasis (HAT) and Leishmaniasis, respectively, caused by kinetoplastid parasites *Trypanosoma brucei* (*gambiense* or *rhodensiense*) and *Leishmania* spp, closely related to *T. cruzi* [62]. 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 [61]. 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 transilluminator for improved performance. In a recent case report, LAMP blood detection of *T. brucei rhodensiense* was shown [63]. Upon larger-field studies, this methodology could definitely be a major breakthrough towards HAT control [61]. Several studies are also applying LAMP for Leishmaniasis clinical diagnosis [56,64,65]. A recent work with clinical samples (blood, saliva, and tissue) from just two patients showed that the method could be used with crude samples uncompromising sensitivity compared to qPCR, as far as samples were boiled previous to LAMP [64]. Sample boiling preparatory step has been described for *Schistosoma haematobium* LAMP assay too [60].

In contrast, the only reference of a LAMP method for Chagas disease diagnosis is Thekisoe and coworkers published LAMP method, which was developed to discriminate between *T. cruzi* and *T. rangeli* infections in field collected *Rhodnius pallescens* vectors [54]. 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 with 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 [54]. In comparison to the abovementioned 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, an 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 [33,66]. Currently, based on Eiken Co. LAMP technology design, FIND and collaborators, among which is Alejandro Schijman laboratory at INGEBI, set up a *T. cruzi* LAMP assay targeted to the highly repetitive satDNA sequence [67]. The amplification reaction takes 40 min at 65ºC and a subsequent 5 more minutes at 80ºC 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 [67]. The assay showed very good inclusivity and selectivity as DNA from *T. cruzi* stocks belonging to the six DTUs was detected (Figure 1) [67]. 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 *T. cruzi* epimastigotes. In terms of clinical diagnosis, LAMP assay detected congenital and immunosuppressed Chagas disease samples, but chronic patients' samples were only detectable by qPCR (with Ct values below the limit of quantification) [67]. 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 [68]. It provides faster turnaround and works at lower

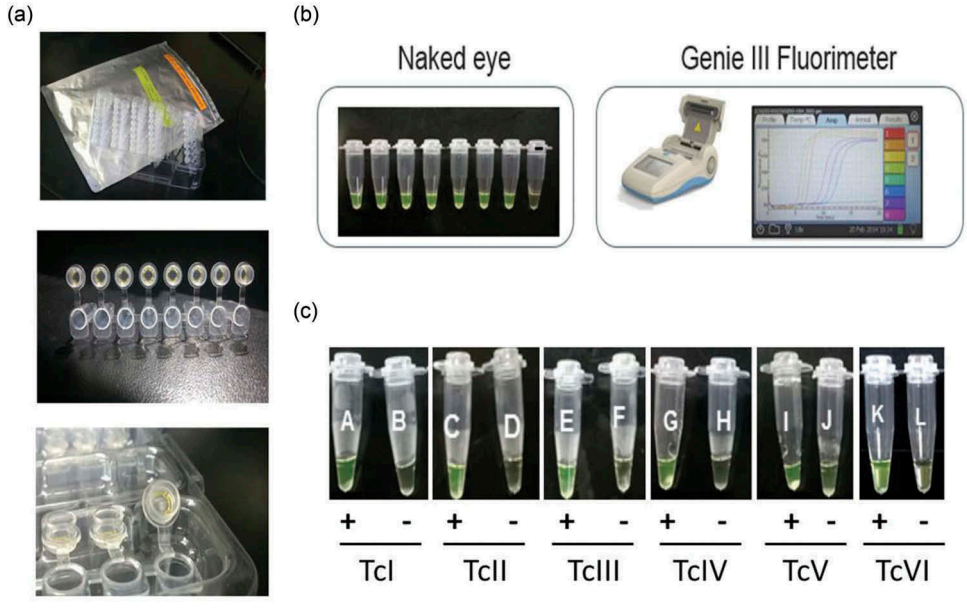

Figure 1. *T. cruzi* loop-mediated isothermal amplification (LAMP) assay. (a) three views of the assay micro-tubes that contain the required reagents dried inside their caps; (b) detection of amplified products directly with the naked eye or using a fluorimeter; (c) assay detected DNA from *T. cruzi* 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. (Japan).

temperature than LAMP [31]. 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 infantum* DNA in dogs [69]. Sample 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 [70]. 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 recharge it [70].

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, TX, USA). Among 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, in which the performance has been shown to match that of commercial thermocyclers at a fraction of their cost [32]. PCR tubes are wire-held from an arm that is coupled to a micro-servo controlled by a programmable microcontroller (Figure 2). 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 and sensitive and provide results within 30 min for target sequences up to 1.5 Kb long [32]. In a subsequent article, TTC capability to detect *Chlamydia trachomatis* DNA extracted from positive urine samples was proved [71]. 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 [71]. Amplification reagents, reactions set up, and tubes required are the same as the ones required for traditional thermocycler-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 acid lateral flow or smartphone-mediated fluorescence detection [72]. Nonetheless, DNA extraction from the samples would still be an issue. In another article, AI Biosciences Inc. team provided a solution for this in the form of a repurposed 3D-printer modified for automated DNA extraction [73]. Even the printer's heated bed heat can be redirected to perform nucleic acid amplification reactions [73].

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 [71] under the terms of the Creative Commons Attribution License © 2016 Chan et al.

The clinical samples detected by TTC were urine of people infected with *C. 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 [74]. Indeed, the use of urine or saliva as samples for POC diagnosis was agreed in the Chagas disease TPP document [33]. In this regard, current efforts undertaken relate to direct detection of parasite antigens in those samples [75,76].

4. Expert commentary

Molecular tests should not be ordered for the clinical management of Chagas disease chronic patients [38,77]. 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 infection 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 trials [14,15]. Furthermore, molecular tests have proved highly sensitive in the detection of acute infections, like those occurring by congenital or oral *T. cruzi* transmission, as well as in anticipating disease reactivation in immunosuppressed patients [6]. More emphasis must be given to its implementation in these particular scenarios, and standardized widespread protocols must be made available to the health-care community. Ideally, the definitive qPCR method should be a single assay rather than a combination of methods to minimize costs. However, if TPP guidelines for Chagas disease diagnostics are considered, 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 [33]. 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) should be pursued. Involvement of the industry in the development of commercial and standardized tests is expected taking into account the high and widespread impact of Chagas disease . These POC 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 women with Chagas disease are infected [78]. Now that blood banks are screened and vector transmission is receding in many regions, congenital route is doomed with ~25% of new infections [48,78]. 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 [2,7,9,78]. In the case of molecular diagnosis in umbilical cord blood samples collected at birth, the detection of *T. cruzi* DNA from maternal origin giving rise to a false-positive result cannot be discarded [78]. 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 sample from the newborn [7]. Nowadays in endemic areas, sampling and testing at birth by micromethod is generally performed to ensure the adherence of the mothers to the health follow-up protocol. Nonetheless, micromethod has been shown to be less sensitive than PCR and to provide a slower results turnaround [7,9]. Even though being made at first month of age, generalization of molecular diagnostics would still reduce the time-to-treatment window and thus increasing the chances to positively respond to it. Definitely, early diagnosis of congenital transmission could be simplified using POC molecular methods.

5. Five-year view

With currently available tools, Chagas disease diagnosis and treatment is barely reaching a fraction of those infected. Diagnosis algorithms must take into account the distinct clinical settings and the field conditions found in many Chagas disease-endemic regions. Therefore, in order to make diagnosis (and treatment) available to more people, low-cost POC diagnostics, amenable in low-resource settings, have to be widely implemented in the territory. Importantly, these methodologies should involve minimal interventions and ideally work with POC samples, such as urine [33]. Unfortunately, use of this type of sample is still largely unexplored and today limits to the detection of parasite antigens in it [75,76]. More research efforts should be placed on the matter.

Molecular diagnosis has been shown to provide an earlier diagnosis of congenital infection than current methods [7,9], but 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 [78]. By the time a serological diagnosis is achieved, a precious time for treatment may have been lost. Therefore, congenital transmission is definitely a diagnostic scenario where easy-to-use molecular tools, such as LAMP or RPA, could play a major role and deserve to be investigated.

In comparison to the serological diagnosis of chronic patients where several RDTs are now available [29], the landscape of POC molecular diagnostics for Chagas disease looks flat. Nonetheless, given the complexity and costs of present PCR methods, their arrival is impatiently expected. Then, towards the establishment of the best diagnostic strategy, population-based studies will need to be performed to show that these alternative methodologies work at least as good a currently available impractical molecular diagnostics.

Key issues

- Chagas disease affects 7 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. As a result barely 1% of infected people receive treatment. Furthermore, diagnosis often arrives when symptoms are advanced and available drugs are less efficient or useless.
- Diagnosis of chronic Chagas disease is performed serologically. Molecular diagnostics are very useful for early detection of congenital infection, assessment of infection reactivation in immune-suppressed patients, and assessment of drug response. In all cases, seropositive status of patients precludes the use of serological diagnostics.
- Standardization of currently available molecular procedures is paramount for reliable comparison of study results. Recent international multicenter efforts have been conducted to canalize diversity into a few best performing assays.
- Molecular diagnosis of congenital infection transmission is not yet widely distributed despite it is faster and more sensitive than current parasitological detection methods. Nonetheless, the complexity and costs of presently available molecular methodologies preclude their generalized use in endemic regions.
- 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.

- Towards an affordable field deployment of molecular diagnostics, the use of new methodological approaches should be implemented. LAMP, RPA or TTC represent potential alternatives to currently impractical methodologies. The hacked 3D printer by AI Biosciences Inc. could be coupled to LAMP, RPA or TTC to provide them with purified DNA and support its amplification within the same low-cost apparatus.

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Declaration of interest

J. Gascon, M. Gallego and A.G. Schijman are members of NHEPACHA scientific network. J. Gascon is a member of the scientific communities of CEADES (Bolivia) and ISGLOBAL. J. Alonso-Padilla's position at ISGLOBAL is funded by Instituto de Salud Carlos III RICET Network for Cooperative Research in Tropical Diseases (RD12/0018/0010 ISCIII; MICINN, Spain). M. Gallego is Professor at the Faculty of Pharmacy of the University of Barcelona and Associate Researcher at ISGLOBAL. Currently, M. Gallego is supervisor in the EU funded Euroleish training network. A.G. Schijman is director of LABMECH and member of the Directory of INGEBI. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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