Title: Practical diagnostic algorithms for Chagas disease: a focus on low resource settings.

Abstract (200 words max)

Introduction: Chagas disease, caused by the parasite *Trypanosoma cruzi*, is the neglected disease with the highest burden in the Americas. Two drugs are available for treatment, but access to them is challenging due to complex diagnostic algorithms. These are stage dependent, involve multiple tests, and are often ill-adapted to the reality of vast areas where the disease is endemic. Molecular and serological tools are commercially available to detect acute and chronic infections, with the performance of the latter showing geographic differences. Recent breakthroughs in the development of new diagnostic tools include the validation of a loop-mediated isothermal amplification assay for acute infections (*T. cruzi*-LAMP), and the regional-level assessment of several rapid diagnostic tests (RDTs) for chronic infections, with the aim to simplify testing, especially in resource-limited settings.

Areas covered: This review outlines the existing algorithms, and proposes new ones focused on point-of-care testing to improve usability and accessibility.

Expert Opinion: Integrating point-of-care tools into existing algorithms through the use of *T. cruzi*-LAMP and RDTs will increase access to timely diagnosis and treatment. However, additional research is needed to validate the use of these techniques across a wider geography, and to better understand the cost-effectiveness of their large-scale implementation.

Keywords: Chagas disease; *Trypanosoma cruzi*; diagnosis; ELISAs; RDTs; PCR; LAMP; point-of-care.

1. Introduction

Chagas disease is the most prevalent neglected tropical disease (NTD) of the Americas, affecting over seven million people, and causing between 9,000 to 12,000 deaths every year.[1] It is estimated that up to 70 million people are at risk of becoming infected with its causative agent, the kinetoplastid parasite *Trypanosoma cruzi;* which is mainly transmitted by insects from the *Triatominae* subfamily.[2] Other forms of transmission include blood transplant, organ donation, and congenital infection. The latter accounts for 20% of newly diagnosed cases,[3] and represents the most common transmission route in non-endemic areas.[1]

The disease is characterized by two distinct stages. The initial acute stage lasts about 8 weeks and is mostly asymptomatic, or presents as an undifferentiated febrile syndrome.[1] A long-lasting chronic stage that can span several decades follows the acute one. It is estimated that 30-40% of those chronically infected will develop life-threatening cardiac and/or digestive manifestations, which can include cardiomegaly, arrhythmias, and ventricular dysfunction, as well as dilation of the colon and esophagus.[1]

The diagnosis of the disease is dependent on the clinical stage.[4] During acute infection, and given the relative abundance of free-swimming *T. cruzi* trypomastigotes in the peripheral blood of the host, the infection can be detected by parasitological, or more reliably, through molecular methods. Classical parasitological methods depend on the microscopic detection of these forms in blood, and include parasite concentration methods, in which samples are sequentially centrifuged to concentrate the parasites.[4,5] For instance, the micromethod is a concentration assay that requires a small blood volume, collected and centrifuged in capillary tubes or microtubes, and it is extensively used for the diagnosis of congenital infections in endemic areas.[6] Indirect parasitological methods, like hemocultures and xenodiagnosis have also been used to diagnose the acute stage.[7,8] While generally cheap and simple, most parasitological methods are strongly operator-dependent and have low sensitivity (generally below 40%), which entails the use of additional serial tests to reach a diagnosis.[4,5]

Molecular methods represent a much more sensitive, although expensive and technically complex alternative. Different traditional and real-time polymerase chain reaction (PCR and qPCR) in-house and

commercial protocols already exist.[4] Recently, assays based on loop-mediated isothermal amplification (LAMP) or recombinase polymerase amplification (RPA), which do not require expensive equipment, have surfaced as a suitable molecular alternative, especially for low-resource settings.[9,10]

In contrast, diagnosis of the chronic stage relies on serological methods based on the detection of IgG antibodies against parasite antigens, given the generally low and highly variable parasitaemia observed in this stage. A wide variety of conventional serological tests have been reviewed elsewhere,[4] with enzyme-linked immunosorbent assays (ELISA), indirect hemagglutination assays (IHA), and indirect immunofluorescence (IIF) being the most widely used. Recently, chemiluminescent microparticle immunoassays (CMIA) for the detection of anti-*T. cruzi* IgGs have also become available, and are mostly used for blood bank screening due to their increased throughput.[4]

In order to account for the ample antigenic diversity of *T. cruzi*, and to reduce the possibility of false negatives, the World Health and Pan American Health Organizations (WHO - PAHO) recommend the agreement of two serologic tests based on distinct antigen sets to confirm the diagnosis of chronic infections.[5] However, these techniques require reagents and equipment, which are generally only available in reference laboratories of endemic countries. In addition, their turnaround of results can take several weeks, leading to a significant number of losses to follow-up in vast areas distant to those referral laboratories.

In that context, rapid diagnostic tests (RDTs) based on an immunochromatographic assay format, represent a promising alternative. They can provide a qualitative result in less than an hour, require no specialized equipment and can work with a minimal volume of blood that can be finger-pricked.[4] Like recombinant ELISAs, RDTs are also based on recombinant antigens. Several are commercially available, and their performance has been extensively evaluated in some regions, like in Bolivia.[4] Despite this, their use remains restricted to screening, and conventional assays are still required to confirm the diagnosis. In the past few years, the quality performance,[11] and high agreement of the combinatory use of two RDTs to detect infected patients in certain regions of Bolivia,[12,13] Argentina,[14,15] and Colombia,[16] have fueled the debate about the possibility of trusting RDTs for confirmatory diagnosis.[17] Nonetheless, their

sensitivity and specificity have been shown to have major geographic variability,[18–20] and region-specific validation of their performance must precede their implementation.[17] Similarly, arguments in favor of a single-test strategy have been raised due to the remarkably high sensitivity of new generation assays based on recombinant antigens, both conventional serologic tests or RDTs.[17,21]

In this work, we review the current state of tests for the diagnosis of acute and chronic Chagas disease. Whether the use of such tests could support changes in existing diagnostic algorithms is discussed, especially the prioritization of point-of-care (POC) assays, whose implementation might generalize timely access to treatment of patients in resource-limited endemic areas.

2. Molecular methods for the diagnosis of *T. cruzi* infection

2.1 PCR and related techniques

Molecular methods for the diagnosis of *T. cruzi* infection have existed for over thirty years,[22–24] but have only been standardized in the last decade.[25–27] The use of the PCR has improved the diagnosis of acute *T. cruzi* infections, as well as the monitoring of treatment in chronically infected subjects. In fact, a Target Product Profile for the use of molecular diagnostics in both stages has been proposed.[28]

Notably, PCR testing is extremely useful for the diagnosis of children born to *T. cruzi*-infected mothers, as its sensitivity is significantly superior to that of parasitological methods.[29] Thereby, its use has been adopted in some non-endemic regions for the diagnosis of congenitally acquired infections.[30,31] Molecular methods are less reliant on the operator's skill, easier to automate, and yield a higher throughput, attributes that contribute to reduced sample processing times, and make them suitable for standardization.

A recently developed TaqMan real-time PCR has demonstrated a particularly superior performance in the diagnosis of congenital infection around one month after birth using a small volume of peripheral blood (250 μl for DNA extraction), a very important feature for its application in newborns, in which small blood volumes are strongly preferred.[32] Therefore, the availability and widespread use of such highly sensitive techniques will be key in WHO-PAHO's framework for the elimination of mother-to-child

transmitted infections (EMTCT-Plus), which in the Americas include *T. cruzi*, and aims at diagnosing and treating up to 90% of all children vertically infected with the parasite.[33]

There are several qPCR kits on the market, generally validated with samples from subjects from different Latin American countries through collaborations between the public sector and private funders. In general, these studies found a high sensitivity and specificity, as well as a high degree of agreement between the different alternatives, supporting their use over microscopy-based methods.[25] The different primers and TaqMan probes used for the detection of *T. cruzi* DNA have been reviewed elsewhere.[4,34] However, despite having obvious advantages over classic parasitological direct detection methods, the cost of reagents, their supply chain, and the expensive equipment necessary to process the samples, remain important limitations to their deployment in endemic areas.

2.2 Molecular methods for POC diagnosis: isothermal amplification techniques

Emerging technologies have contributed to the development of innovative diagnostic tools for Chagas disease. Among these, the use of POC molecular diagnostics which can be performed at constant temperatures (isothermal amplification) has gained attention for its potential to improve timely and accurate diagnosis of acute *T. cruzi* infections. While there are various formats,[35] loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) are the two that have been studied for the diagnosis of Chagas disease, mostly focused in low-resource settings.[9,36] Both techniques can be carried out in block heaters or water baths at a constant temperature of 65 °C or 40 °C, respectively; precluding the need for expensive thermal cyclers, and thus considerably reducing their associated costs.

Although RPA requires lower amplification temperatures and results are obtained in a shorter time compared to LAMP, only one RPA protocol has been described for the detection of the *T. cruzi* parasite in dog samples, showing a performance comparable to that of PCR.[36] In comparison, two LAMP assays have been evaluated with human clinical samples.[37,38] Specifically, Eiken Chemical company (Tokyo, Japan) developed a *T. cruzi*-LAMP prototype, targeted to a widely used satellite nuclear DNA sequence (satDNA), which is very well suited to POC settings as it carries all necessary reagents for the amplification

reaction dried in the LAMP tubes´ lids. This test demonstrated a sensitivity and specificity equivalent to that of qPCR too, with a near-perfect statistical agreement to this technique for the diagnosis of congenital and oral infections.[9,37] More recently, the use of LAMP has also been proposed for the follow-up of treated patients,[39] although its applicability in this context might be limited due to the natural history of chronic infection, and the low parasitemia observed in both treated and untreated patients. Anyhow, compared to PCR, it would be an easier to use and lower cost tool for the post-treatment follow up of chronically infected patients, at least to ensure treatment failure in case of positive determinations.

In addition to anticoagulated liquid blood, it has been recently shown that LAMP can also be performed with dried blood spots (DBS).[37,40] These samples are collected on filter paper and can be conveniently stored at room temperature. DBS are regularly used to facilitate the sample collection process for the diagnosis of other tropical diseases in resource-limited health centers.[41,42]

The most significant factor limiting the applicability of these techniques is that they still require a DNA extraction step, which can be technically challenging in many POC settings. Several solutions have been considered, including boil and spin extraction,[9] a modified 3D printer to act as a semi-automated nucleic acids purification station,[43] and more recently, an already commercially available kit for ultra-rapid extraction (PURE; Eiken Chemical Co. Ltd., Japan).[40] The standardization and application of at least one of these techniques will be essential for the eventual large-scale deployment of isothermal assays.

3. Serological methods for the diagnosis of *T. cruzi* infections

Different protocols for the detection of IgM antibodies have been proposed for the diagnosis of acute *T. cruzi* infection.[44,45] However, the size of IgM molecules makes this approach prone to nonspecific reactivity, negatively impacting the specificity of these methods.[46] In contrast, serology is the best option for the diagnosis of chronic infections, in which long-lasting levels of specific IgG type antibodies against *T. cruzi* antigens are produced. At present, there are several conventional tests (ELISAs, IIF, IHA),[47] and non-conventional RDTs commercially available (see section 3.3 for more details on the latter).

3.1 Overview of ELISAs and other conventional serology tests

ELISAs are widely used due to their convenience, sensitivity, and specificity. The two main types for Chagas disease diagnosis are based on whole parasite lysate antigens, or parasite antigens expressed by means of recombinant DNA technology. The formers have been widely used over the years due to their ability to detect a broad range of antibodies against *T. cruzi* proteins. Nonetheless, such a broad reactivity can also limit their specificity, as whole parasite lysates can cross-react with antibodies produced by other infections, leading to false-positives, and thus, to a decreased accuracy of results; for instance, in regions where closely related *Leishmania spp.* co-exist with *T. cruzi*.[21,48,49] Therefore, lysate antigen ELISAs are typically combined with other assays to curb this limitation. That is frequently done using ELISAs based on recombinant antigens, which show reduced cross-reactivity with antibodies produced from other infections and better specificity, making them particularly valuable in areas where co-infections occur.[50] The high sensitivity of several ELISA assays from different manufacturers has been validated, while their specificity is more variable, particularly for parasite lysate-based assays.[51–53]

Following a similar principle as the ELISAs, IIF is used to detect anti-*T. cruzi* antibodies in serum or other bodily fluids. After incubating patient samples on microscopy slides or microplates coated with fixed *T. cruzi* parasites or antigens, antibodies against the parasite present in the serum will bind to the antigens on the surface. Then, fluorescently-labeled secondary antibodies are added, and bind to any antibodies primarily attached to the *T. cruzi* antigens, allowing their identification using fluorescent microscopy.[54,55] There are IIF assays commercially available, including kits designed for the detection of anti-*T. cruzi* IgM and IgG molecules.[56] While highly sensitive, IIF is labor-intensive and requires skilled personnel and an expensive fluorescence microscope for its performance and interpretation, making it unsuitable for large-scale screening or in ill-equipped laboratories.[4] Besides, the test is also subjective to a certain extent, and the results may be influenced by the operator's experience and judgment. Thus, it is often used as a confirmatory test in combination with other simpler serological assays.

In principle, IHA assays are the cheapest and simplest conventional serologic tests.[4] In the IHA test, serum samples are mixed with sheep red blood cells (SRBCs) coated with *T. cruzi* antigens. If specific

antibodies against the parasite are present in the serum, they will bind to the antigens on the SRBCs. That interaction will lead to the agglutination of the SRBCs, and the degree of agglutination can then be visually assessed. Studies comparing IHAs with other serological tests have consistently demonstrated the high specificity of the test,[52] although it tends to have a lower sensitivity compared to other platforms. For this reason, it is often used as a secondary test, in conjunction with other assays.[4]

Several studies have compared the geographical performance of these serological tests, describing generally high sensitivities,[57] and good correlation between the results of samples from different countries.[58] Despite this, the performance of serologic methods, including ELISAs, is influenced by the geographical origin of the samples, and shown to be particularly poor with samples from Mexico and other regions of North America,[20,57] where discordance of tests results is relatively common.[59]

3.2. Chemiluminescent Microparticle Immuno Assay (CMIA)

Chemiluminscent tests present a high sensitivity, specificity, and automation capabilities,[60] making them increasingly popular alternatives for the diagnosis of *T. cruzi* infections.[21] In the CMIA test, patient serum samples are mixed with microparticles coated with *T. cruzi* antigens. If specific antibodies against the parasite are present in the sera, they will bind to the antigens on the microparticles. After washing away any unbound antibodies, a chemiluminescent substrate is added, leading to the release of light proportional to the number of antibody-antigen complexes formed. This emitted light is measured by a specialized instrument, and the intensity of the signal corresponds to the presence and quantity of specific antibodies in the sample.[60]

CMIA offers several advantages over traditional serological tests, including a high throughput capacity and operator-independence, given its automated nature. Furthermore, CMIA has demonstrated excellent diagnostic accuracy and reliability,[61] making it a valuable tool for large-scale screening and surveillance programs, like blood bank testing in both endemic and non-endemic regions. Despite its advantages, the use of this technology is mostly limited to high-resource settings, due to the cost of the required equipment.

3.3 Characteristics and performance of RDTs

At least 41 RDTs for diagnosing Chagas disease have been developed (see Supplementary Materials - Table 1), of which 25 (61%) can be obtained commercially for diagnostic use. The latter are summarized in Supplementary Materials Table 2, together with their performance validation details. The remaining 16 tests are intended for research use only (n = 2), do not have a known commercial status (n = 2), were revoked (n = 9), or are still in development stages (n = 3) (Supplementary Materials - Table 1).

All commercially RDTs are cassette or stick immunochromatographic tests, and have a quick turnaround of results, usually less than 15 minutes, with four of them reporting readout times of 10 minutes: Simple Chagas Test/Stick Chagas (Operon, Spain),[62,63] Simple Test WB (Operon, Spain),[63–65] Trypanosoma Detect Rapid Test (InBios International, USA),[12,13,16,19,66,67] and Chagas Test (Veda Lab, France)[68] (Supplementary Materials - Table 2). Most tests are suitable for use at the community level (n = 18/25, 72%), just relying on a few drops of blood as sample, which can be obtained through a finger puncture, and do not need any further processing.

Comparing the clinical performance of different tests is complex, given the lack of a uniformed validation process, which leads to the use of different sample types, volumes and reference tests, with only eight of the tests being compared with the currently accepted two-test gold standard algorithm (Supplementary Materials - Table 2). The performance of nine RDTs has been independently evaluated in clinical studies, most of which took place in controlled laboratory conditions, not necessarily reflecting characteristics of the field, and not always accounting for the previously described regional changes in reactivity. Overall, the most thoroughly researched RDTs are: (i) Chagas STAT-PAK (Chembio Diagnostic Systems, USA),[69–74] (ii) SD Bioline Chagas AB Rapid Test (Standard Diagnostics, Korea), [14,15,65,75–77] (iii) Chagas Detect Plus Rapid Test (InBios International, USA),[12,13,16,19,66,67] and (iv) WL Check Chagas Test (Laboratorios Wiener, Argentina).[14,15,65,75,78]

Remarkably, among the 41 retrieved RDTs, we only found specific information regarding the recombinant antigens they were based on for six of them. This information is essential for the eventual

selection of more than one RDT in case of combined use, in compliance with WHO - PAHO recommended algorithm. For a full description of commercially available RDTs see Supplementary Materials – Table 2.

RDTs have been used for screening purposes in countries like Bolivia,[12] Colombia,[16] Argentina,[15] and Paraguay,[79] always followed by confirmation of their results with a conventional serological test. In clinical studies investigating the combined use of RDTs as an alternative to those, for instance in areas of Bolivia, RDTs based on different antigenic sets evidenced a sensitivity of 100% and a specificity of 99.3%, compared to the use of three conventional serological tests performed at a reference center.[12] Similar results were obtained in a study in Northern Argentina, where dual RDTs reported a combined sensitivity of 97.4% and a specificity of 100%.[15] In another work made in Colombia, RDTs sensitivity and specificity were above 99% for the two tests evaluated.[16] Altogether, these studies feed the debate about relying on RDTs for confirmatory diagnosis.[17]

Nevertheless, as it has also been evidenced for conventional serological tests,[20] a major limitation of RDTs is their variable performance with samples from different geographic settings.[18–20] Such a phenomenon is explained by the diversity of *T. cruzi* strains from one genotype or another predominantly circulating in different geographical regions, as well as to the prevalence rates and population genetics influencing the immune responses of the infected individuals.[80] This confirms that a local validation of their performance should always be made before they are implemented for screening or diagnosis in a certain region, which is anyways very much the same that must be done with conventional tests.[17]

Another challenge to circumvent, more related to marketing and distribution, relates to the low economic incentives behind producing RDTs for Chagas disease, with manufacturers regularly raising issues regarding the low expected price of tests in comparison to those used to diagnose other conditions. The market for Chagas disease RDTs is fractured, with a surprisingly large number of manufacturers, some commercializing the same test under a different presentation (Supplementary Materials - Table 2). This highlights the need to prioritize specific products and manufacturers, based on their performance validation, and the adaptability of their products to include different antigens, according to their region of intended use.

3.4 Applied bioinformatics for the design of diagnostics

In an attempt to design innovative tests that are functional in the widest possible geographical area, computational approaches can be used to identify conserved parasite antigens, or epitopes within these antigens. One essential computational resource for studying *T. cruzi* is TriTrypDB, part of the VEuPathDB Bioinformatics Resource Center.[81] TriTrypDB currently hosts a variety of sequence data from 31 *T. cruzi* strains, allowing researchers to access and analyze these datasets freely. This greatly facilitates the identification of potential antigens that are conserved within different strains, and across different DTUs. Moreover, sequence analysis can help uncover conserved regions within these putative antigens, and computational tools used to predict epitopes within them.[82] Theoretically, since these epitopes should be shared by all *T. cruzi* strains, they would be suitable targets for an accurate diagnostic test that could be used in different geographical regions.

The reality, however, is more complex, as different *T. cruzi* strains exhibit distinct patterns of gene expression,[83] and the organism undergoes significant changes throughout its life cycle. Additionally, genetic variations and haplotype frequencies in human populations can also impact the immune response generated against the parasite,[82] and therefore, the performance of serologic techniques.

A recent in-depth evaluation of the human antibody responses against *T. cruzi* in six different geographic regions of the Americas found a remarkably diverse immune response to the parasite, with most antigens showing a very low seroprevalence, and being exclusive to each analyzed sample, suggesting a strikingly variable serologic response to the infection.[84] Interestingly, despite this diversity, the immune responses appeared roughly clustered into two main groups: one for North America and northern South America (USA, Mexico and Colombia), and another for southern South America (Bolivia, Argentina and Brazil). This clustering aligns with the current understanding of the human biogeography of the continent, and the natural history of *T. cruzi*,[80] and could help identify useful antigenic sets for the diagnosis of the disease for each region.

Furthermore, within this diverse immune response panorama, researchers identified several antigenic regions with high seroprevalence across the different samples, most of which represented novel

antigens that had not been previously described. To validate the diagnostic potential of these novel antigens, authors tested a selection of them against two panels of samples. In both panels, these antigens successfully detected cases that would have otherwise gone undiagnosed. Notably, the top-performing antigen in both panels, which belonged to the trans-sialidase superfamily, outperformed the Wiener Chagatest, a widely used and validated commercial ELISA diagnostic test.[84] Even though the study focused solely on linear peptides from only two *T. cruzi* strains (belonging to DTUs I and VI), it represents an extremely valuable founding stone likely to accelerate the advancement of novel serologic diagnostic tools.

4. Current *versus* alternative diagnostic algorithms

As previously mentioned, diagnosis of Chagas disease is dependent on the phase of the infection (acute or chronic). In both cases, current diagnostic algorithms involve multiple tests, and may span several weeks, or even months for congenitally acquired infections, until a diagnosis is reached.[4,5] While this guarantees good specificity and sensitivity, the long turnaround time entails a high risk of losing follow-up of patients before the treatment can start. Moreover, ultimately, both the acute and chronic algorithms require equipment and reagents that are often unavailable in vast areas highly endemic, and where access to regional reference laboratories is complicated by distance, bad communications, sample referral logistics, or socio-cultural features. In consequence, although precise, current algorithms are impractical in most endemic settings.[85]

4.1 Acute stage diagnosis

In regions with endemic transmission of Chagas disease, acute infections should be suspected in patients with undifferentiated febrile syndromes, after other causes, such as arboviral infection, have been discarded. This would be of particular importance if peripheral edema and clinical signs of myocarditis are observed, as these symptoms are typically described in outbreaks following consumption of food or drinks contaminated with the parasite (oral infections).[86] In these cases, the current diagnostic algorithm includes performing direct parasitological detection and/or molecular tests, if available, in peripheral whole

blood samples.[4,5,31] Although serology is not useful for the diagnosis of acute infections, it should be performed to determine the patient's baseline infection status, and if negative, should be repeated between six and eight weeks later, and again after six months, to detect late seroconversion.[31]

On the other hand, direct parasitological tests and/or molecular methods are also recommended for the detection of reactivation in seropositive immunocompromised patients. Depending on the clinical context, these can be performed in blood samples, from unfixed tissue biopsies, or cerebrospinal fluid.[31]

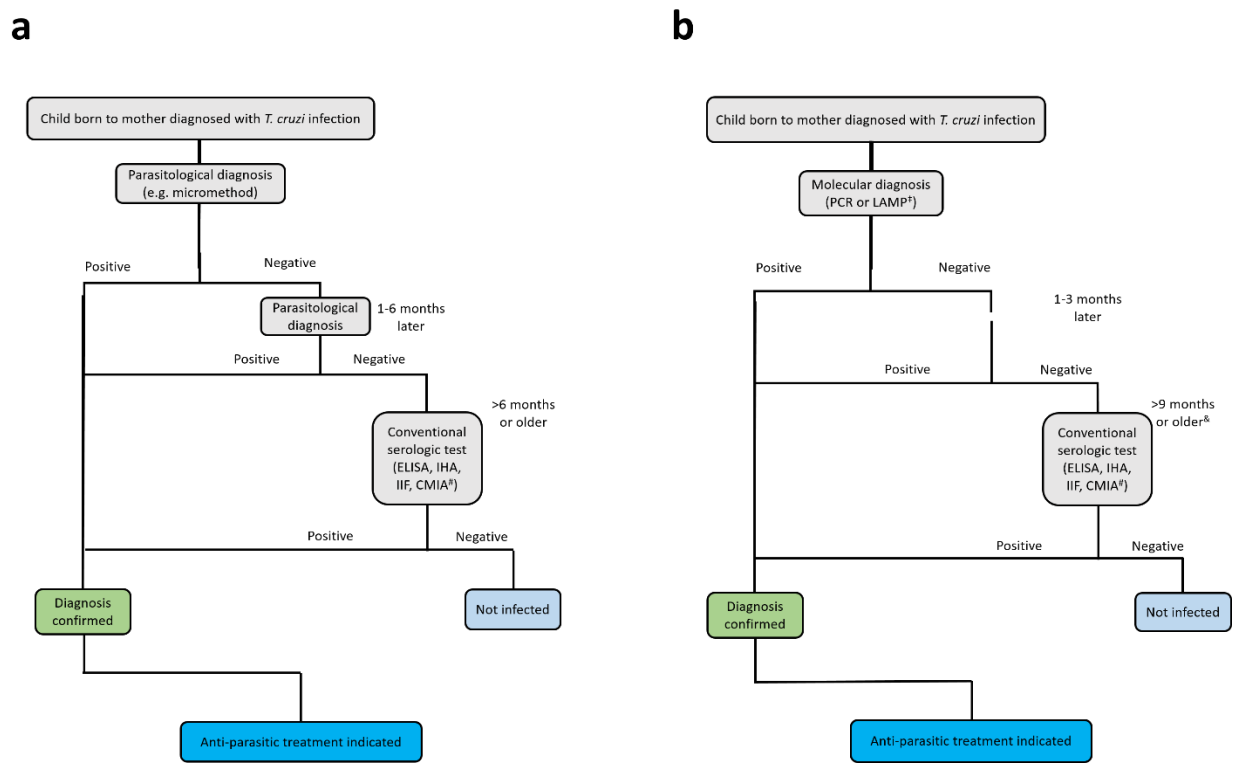
Congenital infections represent a major route of infection in endemic and non-endemic regions,[87] and in this scenario, the current algorithm involves performing direct parasitological tests (e.g. micromethod) at birth and a few months later (Figure 1). If available, a PCR or qPCR should be performed at the same time points, as is regularly done, for instance, in certain screening programs of non-endemic countries like Spain,[88–90] the United Kingdom,[31] and more recently also approved in Argentina. However, given their price and relative complexity, such techniques are rarely used in endemic countries, except in the context of well-funded clinical trials. This makes the late serological study of the children, who might have only been evaluated by suboptimal parasitological tests, particularly important in these settings. Nonetheless, serological testing of congenital infection should be postponed until at least the ninth month of life, given the confounding effect of circulating maternal anti-*T. cruzi* IgGs.[71,91] A positive result in any of the aforementioned tests implies commencing anti-parasitic therapy (Figure 1).

The *T. cruzi*-LAMP prototype by Eiken, which has been extensively evaluated in the last few years,[9,37] is the most technologically advanced POC alternative to PCR tests. The kit includes all the required reagents for the amplification reaction dried up in the lids of the individual tubes, allowing the storage of all of its components at room temperature. The assay protocol is simple, yields a qualitative readout in less than three hours, and its performance has been already clinically studied.[37,39] More recently, the use of DBS samples has been analytically validated yielding LAMP limits of detection equivalent to the use of liquid blood samples.[40] Usability of DBS could prove fundamental, thinking of a widespread deployment of the technology. Notably, a regular use of highly sensitive molecular diagnosis techniques might preclude the need to carry out serological testing of children by nine months, if

demonstrated that its use manages to detect most, if not all, cases of congenital infection in the first two months of life.

Although market pricing, cost-benefit and health economics analyses are pending for this new technology, it is expected that the range cost stays around 10 USD per determination, in the same line of other already available Eiken LAMP products for the diagnosis of malaria and tuberculosis.[92,93] In order to ensure a wider market, and thus be able to keep production costs low, it will be fundamental to validate the use of the prototype continentally in the short-to-medium term, not only for congenital infection, but also for the detection of other forms of acute disease, infection relapse, follow-up of treated patients, or even for the entomological surveillance of vectors´ gut contents acknowledging molecular detection in this context also yields higher sensitivity than microcopy-based observations.

Figure 1. Existing and proposed algorithms for the diagnosis of congenital infection with *T. cruzi*.



Current diagnostic algorithm for acute infections **(a)**. Proposed alternative algorithm replacing parasitological methods for molecular tests **(b)**. #Although LAMP might be particularly useful as a POC

method in endemic-regions, it could also be useful in non-endemic areas contributing to save costs. ‡The use of molecular methods (PCR or LAMP) might preclude the need to carry out the serological study once maternally derived anti-parasitic IgGs wane.

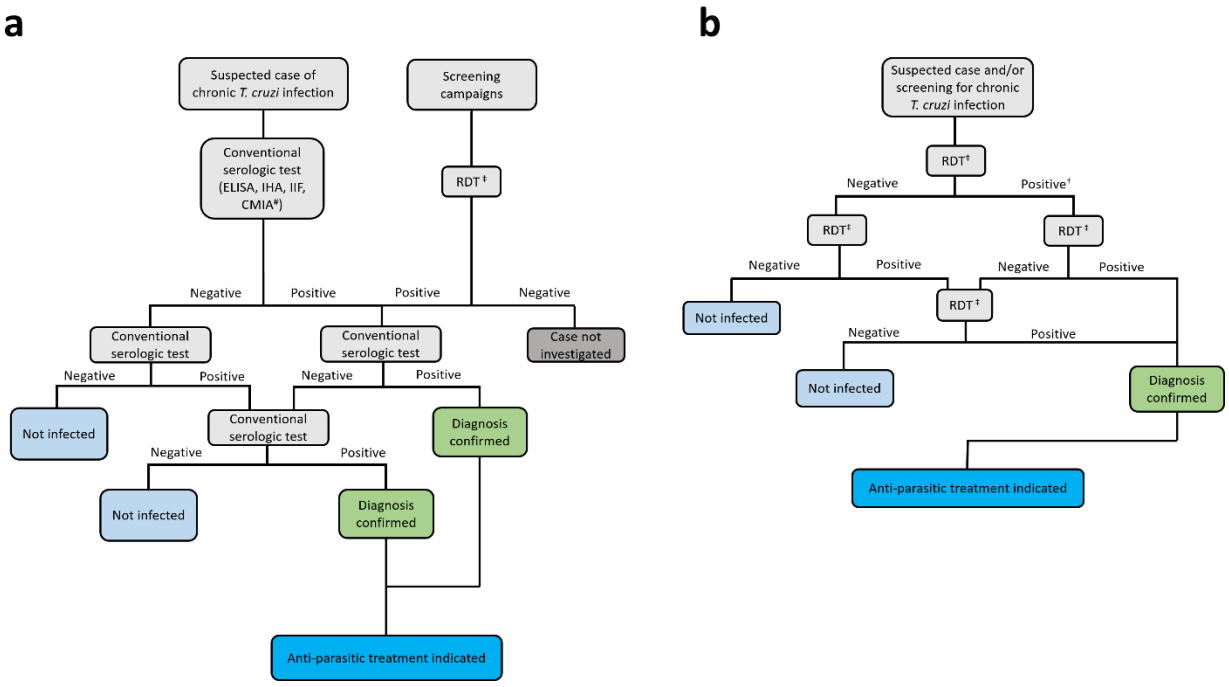
4.2. Diagnosis of chronic infections

As mentioned before, following WHO - PAHO guidelines, at least two tests with different antigen sets should be performed to diagnose chronic infections.[5] The use of ELISA and CMIA, both based on recombinant antigens, represents the ideal combination, given that they provide the best performances.[4] Nonetheless, other methods such as IIF and IHA, the latter being the cheapest and often the only one available in many settings, are most commonly coupled to the ELISA. In case of conflicting or undetermined results, a third test should be performed. All these assays work with serum or plasma samples that require venous blood extraction and centrifugation. Moreover, reagents and samples must be kept refrigerated, complicating transport and storage logistics. These features can increase the cost of the current diagnostic algorithm, limiting its usability in many endemic settings with scarce resources.

Unlike conventional serologic tests, RDTs work with a tiny volume of finger-pricked blood, their result turnaround time is shorter than that of conventional tools, and they do not require a cold chain. Thus, an algorithm based on their combined use for confirming chronic *T. cruzi* infections would represent a breakthrough towards improving access to diagnosis, and treatment, especially for people living in areas distant from well-equipped laboratories. This idea is supported by the remarkably good performance and agreement evidenced by the use of two different RDTs in clinical studies from Argentina,[15] Bolivia,[12] and Colombia,[16] as discussed above. Nonetheless, it is also important to be aware of the limitations of this approach, mostly represented by the geographically diverse performance of RDTs.[18,94] Despite this, upon a robust local validation of their performance, the adoption of new algorithms prioritizing their use could prove extremely positive (Figure 2). For instance, RDTs would be particularly useful for the timely detection of chronic infection in pregnant women. This is a population at high risk of loss to follow-up, and failure to diagnose promptly compromises the treatment of their children in case diagnosed infected, which is extremely efficacious at that stage.[95]

On the other hand, emerging evidence from regional clinical studies suggests that it might be possible to rely on a single-test strategy to detect chronic *T. cruzi* infections. Arguments in favor of this statement are based on the remarkably high sensitivity of new generation assays based on recombinant antigens.[17,21] Such an approach, which represents the diagnostic norm in many other epidemiologically similar conditions, could significantly contribute to saving costs, and improve diagnostic capabilities in endemic countries. The implementation of a single-test strategy should be preceded by extensive local validation of the method to be deployed. While further research and expert consensus are necessary, we consider it is a possibility worth discussing, in light of the almost perfect agreement between serological tools obtained in some regions.[12,13]

Figure 2. Existing and proposed algorithms for the diagnosis of chronic *T. cruzi* infection.



Current diagnostic algorithm for chronic infection **(a)**. Proposed alternative algorithm prioritizing POC testing **(b)**. #CMIA is typically limited to use for blood bank screening and is only available in reference laboratories of endemic and non-endemic countries. ‡RDTs, same as ELISAs, should be previously validated in the region of intended use. †Given the very high level of agreement between tests in some regions, in contexts where a second RDT is not immediately available the possibility of starting treatment following a single positive RDT should be considered.

5. Conclusion

Molecular techniques based on isothermal amplification, and RDTs comply with the REASSURED criteria for ideal POC diagnostic tests (Real-time connectivity, Ease of specimen collection, Affordable, Sensitive, Specific, User-friendly, Rapid, Equipment-Free, and Deliverable to end-users).[96] By meeting these criteria, both platforms offer a promising solution for decentralized diagnosis of acute and chronic *T. cruzi* infections in resource-limited settings. Molecular POC tests seem particularly useful in the context of congenital disease transmission, while serology-based RDTs shall increase the number of patients chronically infected with *T. cruzi* who can start anti-parasitic therapy before becoming symptomatic. Regarding these RDTs, while the geographic diversity of currently available antigenic sets is a major limiting factor, recent advances in the characterization of region-wide serologic responses to *T. cruzi* are likely to facilitate the development of more robust tests, or at the very least, provide a list of antigens likely to work in different specific geographic settings.

The incorporation of POC-oriented diagnostic platforms into existing algorithms for the detection of Chagas disease will facilitate access to diagnosis, and to timely and appropriate treatment of a larger number of patients, thus contributing to reducing its burden.

6. Expert Opinion

Chagas disease is estimated to affect over seven million people across the Americas, but the vast majority of cases remain undiagnosed, and therefore, untreated. In part, this can be attributed to the disease's silent clinical progression, which delays treatment-seeking behaviors until irreversible manifestations become apparent. This is worsened by the complexity of current diagnostic algorithms, which involve multiple tests that can span over weeks or months.

The implementation of the LAMP isothermal molecular amplification technology, and the increased availability of immuno-chromatographic RDTs based on recombinant *T. cruzi* antigens, represent alternatives to help close this gap. These innovative tools provide a remarkable opportunity to simplify the

diagnosis of acute and chronic infections, certainly increasing the number of people receiving timely diagnosis and treatment.

Some of the obstacles challenging such a transition have begun to be addressed recently. For instance, fast and simple protocols requiring minimal equipment have been described to facilitate DNA extraction in resource-limited settings, making it technically possible to perform LAMP assays there. On the other hand, the antigenic diversity of *T. cruzi*, and the serologic responses mounted against it, interferes with the performance of conventional serological tests and RDTs. To circumvent it, recent advances in the characterization of the reactivity of continent-wide samples against the proteome of two highly prevalent parasite DTUs, provides a valuable resource for the identification of region-specific and/or globally prevalent antigens.[84] Anyhow, these technological breakthroughs must now be followed by thorough health economics analyses that shed light on the cost-benefit balance of updating existing algorithms.

In light of the high number of studies validating the performance and implementation of these tools in specific geographic regions, like the Bolivian and Argentinean Chaco, pilot programs incorporating the use of alternative algorithms based on LAMP and RDTs should be put in place in these places. We anticipate that this decision will lead to significant increases in the number of people diagnosed and treated, and to reductions in the local burden of Chagas disease, hence providing a very needed evidence to carry on with similar approaches in other regions of the Americas.

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Declaration of interest

The authors declare no competing interests.

Author contributions

All authors contributed to the first draft of the manuscript and further revision. N.M.-P., E.E. and J.C.G.-F created key figures and tables for the paper. All authors agreed on the final version of the manuscript.

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