Title: How effective are rapid diagnostic tests for Chagas disease?

Structured abstract
Introduction: Diagnosis of chronic Chagas disease relies on the agreement of two conventional serological tests based on distinct antigens. Those require cold to preserve reagents and samples, and of equipment and trained personnel to run them. Moreover, results turnaround may delay for several weeks risking loss to follow-up of infected subjects, summoning major disadvantages to access diagnosis (and treatment) in many highly endemic areas.
Areas covered: Recent studies have shown versatility of rapid diagnostic tests for the detection of chronic *Trypanosoma cruzi* infections in referral centers and in field campaigns, with a performance equivalent to that of conventional tools. Remarkably, RDTs do not require cold storage and provide results within an hour. Additionally, they are easy-to-use and can work with a tiny volume of finger-pricked whole blood. Altogether, major advantages towards generalizing their use as alternative to conventional tests.
Expert opinion: Already in 2021, only a small percentage of *T. cruzi*-infected people is diagnosed and treated. Unsuitability of currently used diagnostics, and of the recommended algorithm, to the conditions found in many regions does not contribute to fill this gap. RDTs stand as a promising solution, even though geographical validation should precede their implementation**.**

Keywords: Chagas disease; *Trypanosoma cruzi*; chronic infection; serological diagnosis; ELISA; RDTs.

Article highlights:

- The use of conventional serological tests for the detection of chronic *T. cruzi* infections is not practical in many highly endemic regions with low-resources. This is mainly due to scarcity of appropriately equipped laboratories and trained personnel, and to the current delay in results turnaround that risks the loss to follow-up of infected people.
- Rapid diagnostic tests (RDTs) were developed as an easy-to-use alternative to conventional tests. They do not require cold-chain, can work with a tiny volume of finger-pricked whole blood, and very importantly, they provide results in less than one hour.
- Although performance of RDTs is geographically variable, likely related to the predominant circulating strains and the infection prevalence, recent studies in Argentina and Bolivia have pointed out their high sensitivity and specificity. Joint to their capacity to be used in field screening campaigns, even outdoors, make of them incredibly valuable tools for the mass screening of chronic Chagas disease.
- If proved to work as good as conventional tests upon regional validation, RDTs should be used for the diagnosis of chronic *T. cruzi* infections. A more widespread use of these tools will promote access to diagnosis in many regions where this is now hindered, which could result as well in an increased access to treatment and thus an improved control of Chagas disease.
- WHO / PAHO recommended algorithm for the diagnosis of chronic *T. cruzi* infections entails the agreement of at least two tests based on different sets of antigens to convey a conclusive result. This doubles the diagnosis costs and has

an impact in the time to communicate the outcome. The very high sensitivity and specificity rendered by currently available tests suggests that such recommendation could be re-visited.

Introduction

Chagas disease is a neglected zoonosis caused by the protozoan parasite *Trypanosoma cruzi.* It affects more than 6 million people worldwide, the majority of them in the Americas, where the vectors that transmit the infection are endemic [1]. In recent decades, the impact of the disease has spread to non-endemic regions too due to migrations and vector-independent transmission routes like mother-to-child vertical infections [2].

There are two trypanocidal drugs available to treat Chagas disease: benznidazole (BNZ) and nifurtimox (NFX). They have a very good efficacy when administered early in the initial acute phase of the disease, especially in neonates under the age of one year, who also tolerate the treatment very well [3]. However, this acute phase is mostly asymptomatic and often goes undiagnosed and thus untreated. If host immunity and/or treatment do not clear the parasite during it, the infection becomes chronic. By then, it is estimated that about a third of those chronically infected will develop cardiac, digestive or cardio-digestive tissue damage, which can be life-threatening [1]. In the chronic phase the efficacy of the anti-parasitic drugs is diminished, and their long-term administration regimens involve frequent side effects [4]. Yet, until more efficacious and safer drugs, or new regimens of already existing ones become available, BNZ and NFX are the best and only options. Moreover, despite their limitations, results of several observational studies with chronically infected adults conclude that treatment when clinical symptomatology is absent or incipient has benefits [5, 6].

Anyhow, any medical prescription of treatment must be preceded by a diagnosis report. Regardless of the issues associated to the production and distribution of anti-*T. cruzi* drugs [4], without diagnosis it is not possible to access to treatment, neither it is possible to generate demand for it. Problem is that current algorithm to diagnose *T. cruzi* infections is not practical in many regions where Chagas disease is highly endemic and only count with limited resources.

At present, diagnosis of the chronic phase relies on the agreement of two conventional serological tests, i.e. enzyme-linked immunosorbent assays (ELISAs), indirect hemagglutination assays (IHA) or indirect immunofluorescence (IIF). They all have in common that work either with sera or plasma, which involves sampling by venous puncture to obtain a few milliliters of whole blood and a step to segregate the sera/plasma from it. Both types of sample and the reagents of aforementioned conventional assays that use them require cold storage; and at least the results of ELISAs and IIF, which provide a better performance than IHA, have to be obtained with expensive equipment that need regular maintenance and electricity to work. Moreover, laboratory protocols are complex and must be performed by specifically trained personnel who are scarce in low-resources settings. But, even if all these hurdles were solved, there is yet a major inconvenience associated to the use of conventional tests, and to the fact that two of these must be performed: delay in the turnaround of results. This is a crucial feature, considering the geographical characteristics of many

highly-endemic regions, which are vast in surface but limited in the number of referral health centers, and inhabited by low income populations who cannot stop working to travel to the health-center. As a consequence, diagnosis results are often not returned on time to the patient who is lost to follow-up, only to be possibly encountered years later with ongoing tissue damage, then representing a larger burden to the health system.

RDTs were developed to operate as conventional serology substitutes in those regions [7]. They are easy-to-use and do not need cold for storage. Moreover, some of them can use as sample a tiny volume of whole blood collected by finger prick without compromising their performance [8–10]. Plus, based on recombinant antigens, same as last generation ELISAs [11], many of them yield a high sensitivity and specificity rates [12]. Lastly, a remarkable characteristic of most RDTs is that they have a much shorter turnaround of results than conventional tools [12]. While an ELISA, IHA or IIF can take several hours, some RDTs can return results within half an hour, which means that the person waiting for a response would have a diagnosis in the same visit and could eventually start treatment that day. These are major advantages to prompt their widespread use in order to generalize access to diagnosis, and thereafter access to treatment.

Can RDTs really be an alternative to conventional serological tools?

First reports on the use of RDTs for the detection of anti-*T. cruzi* immunoglobulins appeared in the literature during the early years of this century [13, 14]. Their development responded to the need of easy-to-use diagnostics for field surveillance studies and screening of blood banks. Fifteen years after those initial articles, a recent systematic review and meta-analysis evaluated the performance of RDTs used in a series of studies that complied with being prospective, and adequately comparing them to the standard algorithm based on two conventional tests [15]. Its conclusion was that RDTs performance was on average very good, especially in endemic areas, endorsing a further and more widespread applicability [15].

Before the publication of the systematic review, the combined use of two RDTs for a conclusive diagnosis of chronic *T. cruzi* infections had been explored as an alternative to currently recommended algorithm. Same as the latter stands on the agreement of two conventional serological assays that are based on distinct antigen sets [16, 17], the use of two RDTs based on distinct antigen sets was proposed [18]. Egüez and co-workers evaluated the performance of Chagas Stat Pak (Chembio Inc., USA) and Chagas Detect Plus (InBIOS Inc., USA) versus three conventional assays in a field study that was made in the Platform for integral care of Chagas disease patients in the city of Sucre (department of Chuquisaca, Bolivia) [18, 19]. The conventional assays were Chagas Polychaco IHA (Lemos Laboratorios, Argentina), and Wiener Chagatest ELISAs v2.0 and v3.0, respectively based on parasite lysate and recombinant antigens (Wiener Lab, Argentina). Agreement between both RDTs was perfect (100%) and their level of concordance to conventional assays in terms of diagnostic efficacy was excellent as the kappa coefficient (κ) obtained upon comparing the outcome of the conventional tests and the RDTs duo was 0.99 (CI: 0.94-1.00) [18]. Notably, in that study both RDTs were performed with a little volume of finger pricked whole blood immediately after its extraction while conventional tests relied on sera, which had to be segregated by centrifugation of a larger volume of blood and stored frozen until used. Results encouraged the performance of another field study in a different region of Bolivia. In

between, the publication of a study made in Argentina comparing RDTs WL Check Chagas (Wiener Labs, Argentina) and SD Bioline ChagasAb Rapid (Standard Diagnostics Inc., Korea) with Chagas Polychaco IHA and Wiener Chagatest ELISA was released [20]. This study used serum samples, which may limit its point-of-care translation, but it also concluded that a simultaneous use of two RDTs could reliably diagnose chronic *T. cruzi* infections as it reported a combined sensitivity (Se) and specificity (Sp) of 97.4% and 100% in comparison to the IHA and ELISA assays [20].

In those two works RDTs were made under controlled conditions, in laboratories equipped to run conventional tests [18, 20]. In contrast, in the study performed in the Bolivian Chaco by Lozano and co-workers, RDTs were mostly used in the form of outdoors screening campaigns [21]. The same RDTs and ELISAs as Egüez et al. were used, respectively with a tiny volume of finger-pricked whole blood and sera. Such combination of RDTs has been shown to be based on different sets of parasite antigens and thus its use as a duo would comply with current WHO / PAHO guidelines for chronic Chagas disease diagnosis [21]. As a novelty, a third ELISA (Chagatek, Laboratorios Lemos, Argentina) and RDT (WL Check Chagas) were included to untie in case of discordancy. This time the agreement between the two main RDTs was nearly perfect ($\kappa = 0.86$), but it was better between the main ELISAs ($\kappa = 0.92$) [21]. Although Chagas Stat-Pack yielded very good results when individually confronted to ELISAs outcome, Chagas Detect Plus did not perform as good as it had in Sucre, particularly in terms of its specificity. This could be due to epidemiological differences between regions, as well as to the fact that in the Chaco study RDTs were not run in a controlled place. The latter might be of particular relevance if the RDT in question does not self-contain all required elements for its use [21]. In any case, results reported were very good and would support a combined use of RDTs for the diagnosis of chronic Chagas disease in the region studied, a vast territory with disperse population and a reduced number of health referral centers.

Very recently, a new study evaluating the combined use of the same RDTs as Mendicino et al. [20], SD Bioline ChagasAb and WL Check Chagas, has been published [22]. RDTs used a small volume of whole blood prospectively obtained from subjects in the Chagas disease endemic province of Salta (Argentina) and non-endemic city of Buenos Aires. Overall, performance of both RDTs, compared to three in-house conventional tests (IHA, ELISA and IIF), was better than it had been previously reported [20]. Despite whole blood was used instead of sera, Lopez-Albizu and co-workers described Se values were 97.2% and 93.4%, and Sp values were 97.7% and 99.1%, respectively for SD Bioline and WL Check [22]. With an almost perfect concordance to conventional serology ($\kappa = 0.93$), results obtained would particularly encourage their combined use in the endemic regions studied [22].

The use of RDTs is already contemplated by health authorities as preliminary screening tool, but not their combined application towards a definitive diagnosis. A conventional serological test must confirm the result obtained with the RDT, so the disadvantages of conventional assays cannot be circumvented. Trained personnel and an equipped laboratory will yet be needed, and the results return to the patient could delay for weeks. However, results obtained with certain RDTs, like Chagas Stat-Pak, would even support using it on its own to achieve a final diagnostic outcome [15]. In the two last field studies performed in Bolivia, Se and Sp calculated upon independently confronting Chagas Stat Pack to the standard serological tools were respectively 100% and 99.3%

[18], and 97.7% and 97.4% [21]. In sight of such reported performances in highly endemic regions, it would be desirable to re-think the diagnosis policy of chronic *T. cruzi* infections there, so that it could be better suited to regional characteristics, and even consider the use of a single test. In truth, given the high level performance of last generation ELISAs [11], current algorithm could also be re-formulated [23].

Limitations to the use of RDTs

Promising results have been obtained when RDTs were evaluated in highly endemic regions where co-circulation of *T. cruzi* with closely related pathogens like *Leishmania spp.* was residual. Nonetheless, there have been also reports of a less accurate performance of RDTs when tested in regions where prevalence of the infection is intermediate to low (< 10%) [24]. Since geographical variability of the RDTs performance has been described, it is highly advisable to regionally validate the tools before using them at a larger scale. In their study, Verani and co-workers also showed how average optical density (OD) differed between subjects from the sites in Bolivia and Peru, correlating a better performance of the RDTs with a higher registered reactivity in the ELISAs [24]. A phenomenon also observed when comparing ELISA titers of true positive (TP) or false negative (FN) RDTs´ outcomes within our study in the Bolivian Chaco (Figure 1) [21], which suggests that sensitivity of RDTs might be inferior to that of ELISAs. In regions where prevalence of the infection is low, re-infections are not common, and antibody levels of seropositive subjects are close to the cutoff of the ELISAs, it may occur that RDTs performance is compromised. So far, the studies systematically reviewed by Angheben and co-workers [15], and others more recently described [21, 22] have shown very good positive and negative predictive values (Table 1). Their capacity to detect true positive and negative samples may be connected to high prevalence rates of the infection in the areas surveilled, and to the geographical origin of the subjects studied with predominant parasite types for which the RDTs were tailored.

In fact, the majority of studies on the use of RDTs have been performed in countries of the south cone where parasite types of Discrete Typing Units (DTUs) II, V and VI are more abundant. It would be of great interest to get a clue of what is the performance of RDTs in regions where DTU I is most common. Given the ample genetic and antigenic diversity of *T. cruzi*, the predominant strains that circulate in the distinct regions will surely have an impact in the performance of RDTs, and of diagnostics in general. For instance, this would be the explanation to the poor performance of several serological diagnostics in Mexico [25-27]. Similarly, results reported by Verani and co-workers on the distinct performance of Chagas Stat Pak and Chagas Detect Plus in Bolivia and Peru could also be due to differences in the antigenic profiles of prevalent strains in each studied site [24]. A feature that would explain too, at least in part, why a slightly poorer performance of those two RDTs was retrieved in Santa Cruz de la Sierra (Bolivia) in comparison to the subsequent studies in Sucre and the Chaco [18, 21, 24]. Ultimately, it would be ideal to have universal Chagas RDTs that can be used from Mexico to the southern countries in South America. This would facilitate their validation and could contribute to save marketing costs. Overall, the development of such tools might be feasible in the near future years thanks to the availability of an increasingly higher number of *T. cruzi* genomes and the computational resources to analyze them.

Coming back to the use of RDTs in regions where leishmaniasis co-exist with Chagas disease more studies are also needed. Understandably, it would be expected that companies commercializing them had analytically evaluated the antigens encompassing their RDTs against samples from subjects infected with *T. cruzi* closely related pathogens. But this might not necessarily be the case as stated in InBIOS Chagas Detect Plus insert [28]. References addressing RDTs cross-reactivity between *T. cruzi* and other pathogens have only tested a small number of individuals. For instance, Luquetti and co-workers found that serum from two patients out of nine with visceral leishmaniasis, and two out of eleven affected by hepatitis B, did react with Chagas Stat Pak [13]. A limitation informed in Chagas Detect Plus insert is its potential cross-reactivity with samples of patients infected with hepatitis C, toxoplasmosis, or syphilis [28]. Reversely, while evaluating a RDT for leishmaniasis based on K39 recombinant antigen, Neto and co-workers reported that only one out of 30 subjects - specifically positive to *T. cruzi* by serology with four methods - was positive to the *Leishmania spp.* K39 RDT [29]. The fact that RDTs mostly carry combinations of parasite recombinant antigens, likely selected on the basis of their highly specificity and sensitivity, may indicate that cross-reactivity should not be an issue. Nonetheless, a more extensive study of potential cross-reactivity issues of Chagas RDTs remains to be done.

A key aspect of any tool for the diagnosis of a neglected infection is its cost. Regarding Chagas RDTs, it is true that the cost per determination may be generally higher than the cost per determination using an ELISA test. At least that would be the straightforward conclusion upon comparing their market prices. The indicative prices per determination of several ELISAs was ranged between 0.57 and 2.25$ in a WHO Report on anti-*T. cruzi* assays dated in 2010 [11], while the price of a single RDT may range between 2 and 6.5$ (calculated respectively for InBIOS Chagas Detect Plus and Chembio Chagas Stat Pak at the study performed in the Bolivian Chaco). However, the real cost of having an anti-*T. cruzi* ELISA result would be closer to 8.5$ per determination as the personnel time must also be considered (personal communication from LABIMED, Cochabamba, Bolivia). Therefore, corresponding costs of using RDTs or ELISAs would not be very different and will not represent a limitation to the use of the former as far as their performance was equivalent to the latter. Further studies on the cost-effectiveness of using RDTs will be very important, and ideally they should take into account costs associated to losing a patient to diagnosis (and treatment) as this might be one of the major drawbacks of the current algorithm in many regions.

Conclusion

Currently available data from four recent studies indicate that the combined use of RDTs could substitute conventional serological tools for conclusive diagnosis of chronic *T. cruzi* infections in the highly endemic regions studied. Two were made in Bolivia, and the other two in Argentina. In the most recently published study from Argentina and the two studies from Bolivia RDTs used whole blood immediately after prospective sampling, thus literally permitting to provide a diagnosis report within one hour. This is of outmost importance considering mobility issues to visiting healthcare facilities for those populations living distant from the diagnostic centers. In all four cases, their combined use yielded sensitivity and specificity parameters above 95%, which should encourage the performance of more studies in a wider range of geographical areas to potentially increase the evidences suggesting their alternative use to conventional assays. Remarkably, such very good performance levels were

individually achieved by Chembio´s Chagas Stat-Pak, which would on its own comply with the desired sensitivity, and would almost reach the target specificity, suggested by Porras and co-workers Target Product Profile for point-of-care diagnostics of chronic Chagas disease [30].

Expert Opinion

Larger efforts and investment must be dedicated to validate the use of RDTs in more regions. Their attributes as point-of-care diagnostics for the detection of chronic *T. cruzi* infections positions them in the frontline towards generalizing access to diagnosis. No doubt this is among Chagas disease research priorities, bearing in mind that it is the gate to receive treatment, and current estimates indicate that a very little percentage of those infected are ultimately diagnosed and treated.

For conclusive diagnosis of the chronic phase of the infection, presently used algorithm recommends that at least two serological tests based on distinct antigens are performed. When a combined use of RDTs has been compared to using conventional serological assays, their level of agreement has been almost perfect. Thus, the substitution of conventional tools for easier to use RDTs would be attainable in those highly endemic regions where this possibility was studied. Whether this is possible in other regions affected by Chagas disease should be specifically evaluated, because RDTs performance has been directly associated to anti-*T. cruzi* seroprevalence rate, and the antigenic profile of predominant parasite types will likely play a role too. Moreover, when regionally addressing the possibility of using RDTs, co-circulation of other pathogens should be taken into account too, in the same manner as it has to be considered for the conventional serological assays.

Among the several advantages of RDTs, their quick turnaround of results stands out. Thinking of their use as point-of-care diagnostics in primary health centers or referral laboratories from distant areas, this is a paramount feature. It would allow informing the individual on the diagnosis outcome within an hour, ideally targeting her/him for anti-parasitic treatment in the same visit. Importantly, risks of losing the patient to follow-up would be largely minimized. Having in mind the medical and societal costs of encountering that patient with advanced clinical symptoms years later, studying the feasibility of applying RDTs to diagnose chronic *T. cruzi* infections, and indeed using them if validated, must be done. Cost-effectiveness studies will probably have the last word for that validation, and they should consider the derived costs of having non-diagnosed subjects. In order to arrive to robust conclusions, it is fundamental that they can count with as many results from field evaluations of RDTs as possible.

A matter that may not be easily computed in those cost-effectiveness studies is the great value of RDTs for rapid screening of pregnant women. An increasing volume of evidence supports the great benefits of diagnosing and treating women at child-bearing age to impede vertical transmission of the infection [3]. In our experience, such diagnosis is often achieved during late pre-delivery visits or soon after it, a moment at which RDTs ease of use and rapidity prove crucial. Moreover, on time detection of seropositive mothers is vital for the diagnosis, treatment and follow-up of newborns. For those same reasons, counting with an algorithm based on RDTs would most probably facilitate the anti-*T. cruzi* screening and follow-up of mothers-to-be.

In any case, whether diagnosis is achieved using conventional tools or RDTs, the recommendation of using at least two tests to diagnose chronic Chagas disease, doubles its costs. Perhaps it is time to consider a policy change in relation to the number of tests needed. If it is true that there are some geographical areas where performance of Chagas disease diagnostics is questioned, there are others where available tools yield very good results. This is mostly thanks to the improvements made to last generation serological assays that rely on parasite recombinant antigens. In light of the always limited resources available for the diagnosis and treatment of Chagas disease, relying on one test would save half of the costs of current diagnosis, freeing resources that could roughly be used to diagnose twice as many people.

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