Chapter

More than a Hundred Years in the Search for an Accurate Diagnosis for Chagas Disease: Current Panorama and Expectations

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Abstract

Chagas disease, or American trypanosomiasis, is a parasitic disease of the Americas. In nature, *Trypanosoma cruzi* is transmitted through various species of triatomine bugs. However, non-vectorial transmission can also occur, such as transmission through blood products or by transplanting infected organs, by vertical transmission, and lately by oral route. Currently, Chagas disease affects approximately 6–7 million people worldwide, and the process of urbanization in Latin America and migratory movements from endemic countries have led to Chagas disease being diagnosed in areas where the infection is not endemic. There are several methods for diagnosing Chagas disease. Some of these are mostly used for research purposes, while others are used in routine diagnostic laboratories. According to the World Health Organization (WHO), chronic Chagas disease diagnosis is based on two serological techniques. To establish a definitive diagnosis, the results must be concordant. In the case of discordances, the WHO proposes repeating serology in a new sample, and if results remain inconclusive, a confirmatory test should be performed. This chapter shows aspects of the diagnosis of Chagas disease, which varies in its sensitivity and specificity, and its use depends on the geographical location, the available resources, and the purpose of the diagnosis.

Keywords: chagas disease, *T. cruzi*, diagnosis, serology, antigens

1. Introduction

The infection caused by the protozoan parasite *Trypanosoma cruzi* leads to Chagas disease, with an estimated 6–7 million infected people and nearly 60 million at risk of infection [1, 2]. Chagas disease ranks among the world's most neglected diseases and is considered to be the parasitic infection with the greatest socioeconomic impact in Latin America, being responsible for an estimated US$ 1.2 billion in lost productivity annually [3]. It is a disease that a century after its discovery still requires appropriate control measures, effective treatment, and especially an accurate diagnosis. This disease is endemic to most countries in Latin America [4], but it has now become more important in other regions. The increasing presence of Chagas in non-endemic areas, as well as the resurgence of the disease in

endemic countries, has been a major focus of attention in recent years [5] and, over the last 40 years, has become a global health concern due to the huge migration flows from Latin America to Europe, United States, Canada, and Japan. In Europe, most migrants from Chagas disease-endemic areas are concentrated in Spain, Italy, France, United Kingdom, and Switzerland (**Figure 1**) [6]. The flagellate protozoa *T. cruzi* is usually transmitted through infected feces and/or urine excreted by triatomines (Hemiptera: Reduviidae) during blood feeding. However, this is a nonlinear phenomenon, as mammals can be exposed to infection multiple times through distinct routes [7]; the main routes of transmission of the parasite are through the insect vector, blood transfusion, transplants of organs, congenital via, orally and it is now reported that the infection is capable of being transmitted sexually (**Figure 2**). In addition, the fact that Chagas disease can be transmitted sexually, along with the migration problems of individuals affected with Chagas disease to countries that were previously not endemic, and travel to endemic countries, has direct implications for public health for the spread of this disease [8], the transmission through vector only occurs in endemic areas for this disease. In non-endemic countries, the main routes of transmission are blood and congenital transmission [9–11]. *T. cruzi* has a high genetic diversity, which is why it has been classified into discrete typing units (DTU): TCI-TcVI, in addition, of a genotype associated with bats (TcBat); this classification was made based on different characteristics such as geographical distribution and clinical manifestations of the disease, among others. As TcI is the most widely distributed DTU and with a wide genetic diversity, it has been divided into domestic and sylvatic genotypes (TcIDom and TcISyl) [12].

The disease presents two phases. In the acute phase, ranging from the time of infection until about 6 weeks after this, patients present a high parasitemia and may show nonspecific symptoms, such as fever and headache. During the chronic phase, which can last up to 30 years, approximately 30% of patients develop cardiac complications such as arrhythmias and cardiomyopathy, and 10% of patients may present intestinal complications, especially constipation, or neurological complications. However, the chronic phase is characterized by the absence of symptoms in most patients (**Figure 3**) [1, 13]. The latter, coupled with the fact that patients can be found in non-endemic areas where the disease is unknown, represents an added difficulty for the diagnosis of infection [14]. The effectiveness of methods for diagnosing infectious diseases depends on their sensitivity and specificity for the unambiguous detection of the presence of the pathogen or the specific host

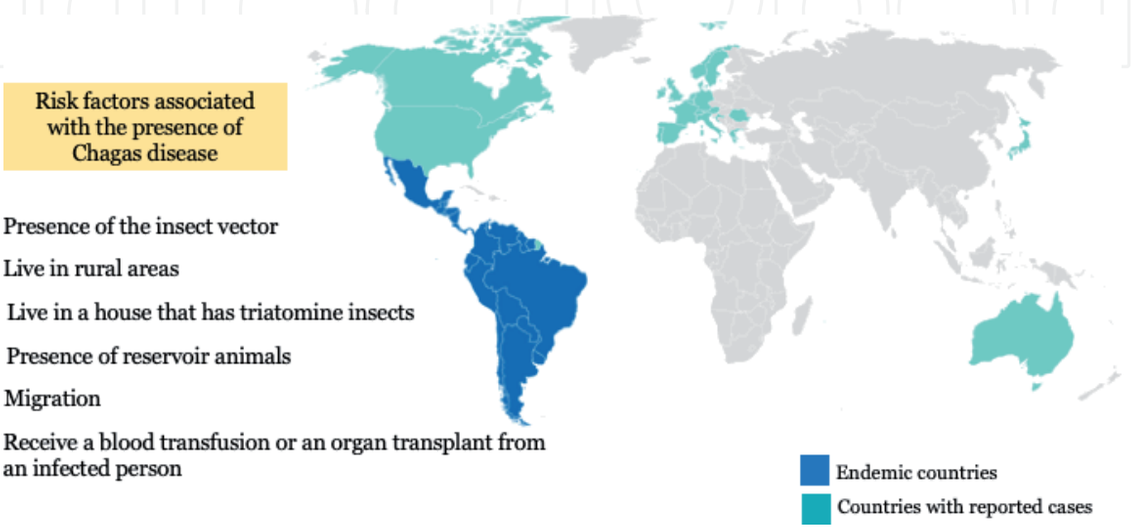

Figure 1.
Distribution of Chagas Disease (WHO: Estimated data 2010).

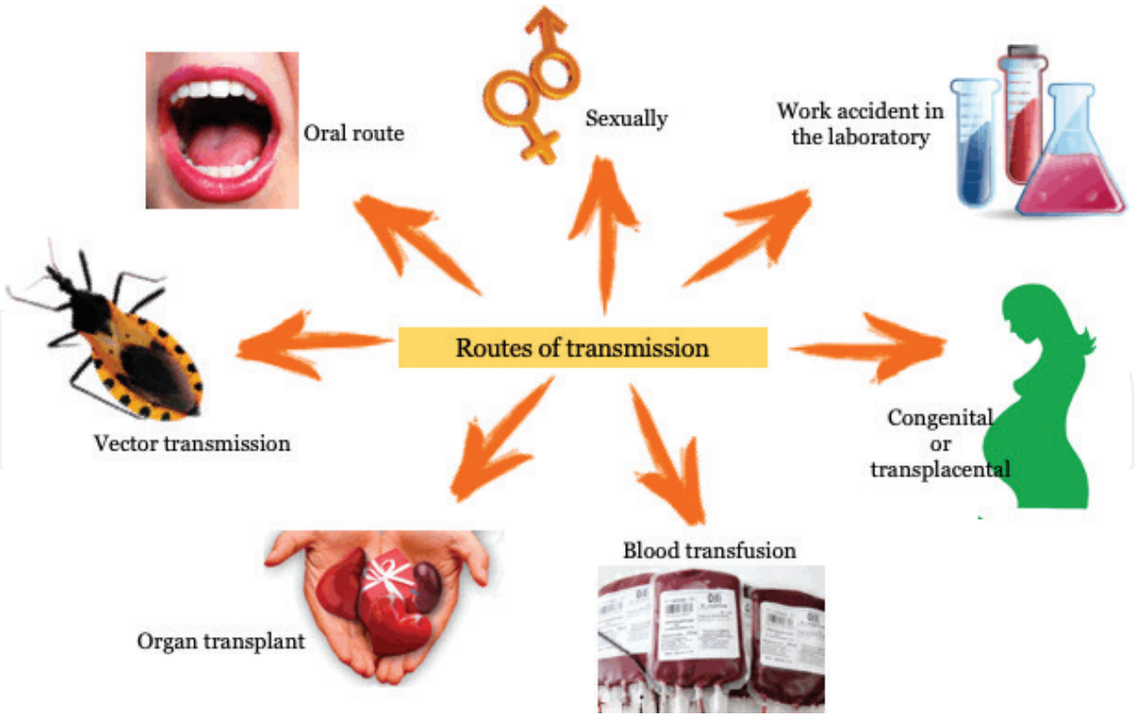

Figure 2.
Main routes of transmission of the Trypanosoma cruzi.

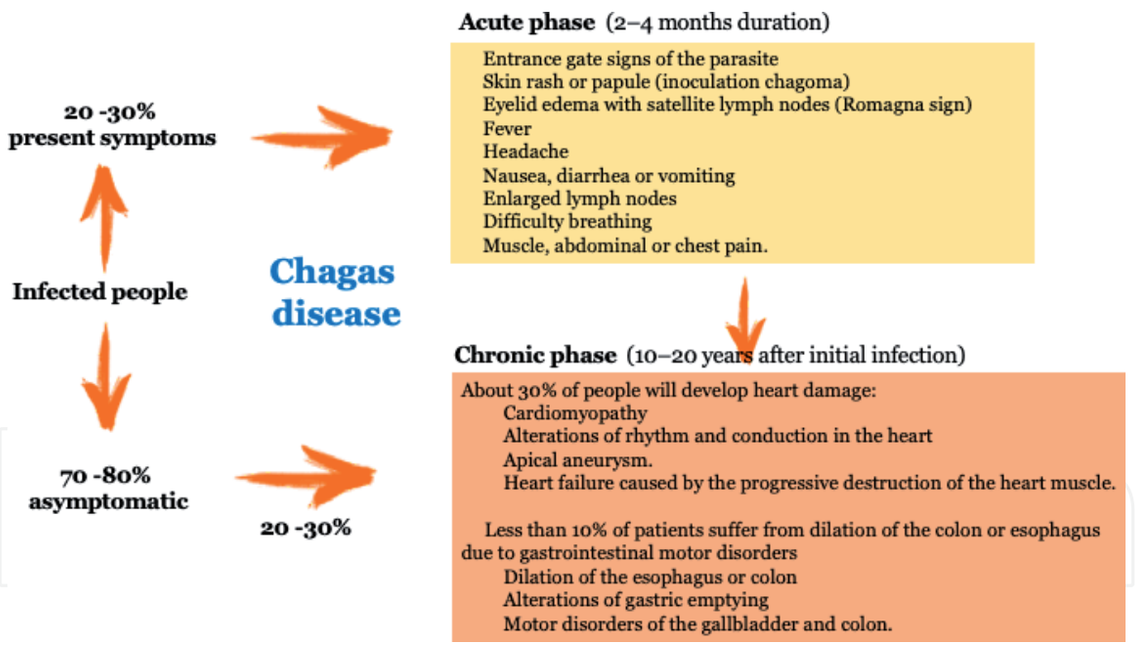

Figure 3.
Chagas disease has two stages or clinical phases: an acute phase and a chronic phase. People (from 70 to 80% of those infected) are asymptomatic throughout their lives, but from 20 to 30% of those affected, this disease progresses to chronic symptoms.

response in response to infection. New technologies based on molecular biology have enabled the identification of biomarkers exclusive of infectious agents, molecules involved in interactions with their hosts, and of the host molecules which mediate response to infection [15]. Infectious diseases remain a major public health problem worldwide. In this scenario, the immunodiagnostic method has been and will remain an essential tool to demonstrate the presence of infection in patients, for disease prognosis, for monitoring clinical studies, and, also, as tools to monitor the success of strategies for control and epidemiological monitoring [16]. Finally,

this chapter was conceived to the current need for an accurate diagnosis for Chagas disease, since the correct diagnosis is a priority not only to identify the people who should receive the appropriate treatment but also to reduce and prevent the risk of transmission through a blood transfusion or an organ transplant.

2. Why the need for an accurate diagnosis for Chagas disease

One of the limitations for the prevention and control of neglected tropical diseases is that the sociocultural aspects associated with diseases are ignored. Cases of Chagas disease in endemic areas occur in specific contexts marked by sociocultural, political, and economic circumstances. In addition, in the case of Chagas disease, the absence of symptoms in most cases, the lack of ability to detect and/or identify the disease, the lack of information on services and immigration policies, affect. It is very important to be able to obtain prevention and control measures for the disease, even in non-endemic countries [17]. Understanding this behavior can allow to guide health policies to combat these types of diseases, where indigenous groups and children are considered especially vulnerable groups.

2.1 The current status of the diagnosis of Chagas disease

Diagnostic methods for *T. cruzi* can be included in three main groups: parasitological, serological, and molecular (**Figure 4**). Parasitological methods aim to visualize the presence of parasites, and their sensitivity varies depending on the stage of infection [18]. For diagnosis of the disease in the phase where the parasitemia is very low, immunological methods (serological) are based primarily on the search for G antibodies (IgG) anti-*T. cruzi* in the blood of patients and their colorimetric reaction visible in the case that the blood of the patients contains the antibodies. [19]. The most commonly used methods are ELISA test (sensitivity = 94–100%, specificity = 96–100%), indirect hemagglutination (HAI, sensitivity = 88–99%, specificity = 96–100%), and indirect immunofluorescence (IFI, sensitivity = 98%, specificity = 98%). Despite being highly sensitive and specific, serological tests can have some cross-reactivity.

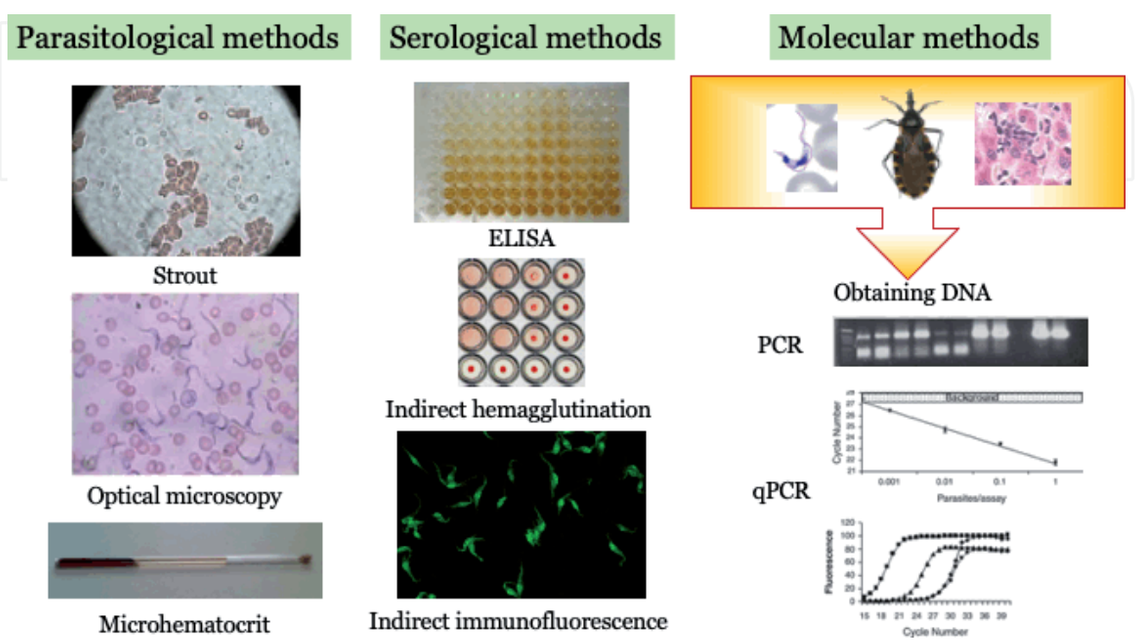

Figure 4.
Diagnostic methods for T. cruzi.

Another technique based on the search for anti-*T. cruzi* antibodies is the Western blot, which has been used in the diagnosis of Chagas disease using mainly excretion-secretion antigens and in some cases recombinant proteins. In the reported works, the Western blot has been used mainly to confirm the serological results obtained when other serological techniques are discordant or when there are cases of cross-reaction with *Leishmania*, and it has been observed that the technique possesses a high sensitivity and specificity. Although this test is not used routinely, and it is not considered as a substitute for conventional serological tests, it may be useful as an additional diagnostic test or for field studies [20–22]. Detection of parasite DNA using molecular diagnostic tools could be an alternative or complement to current diagnostic methods, but its implementation in endemic regions remains limited, due to lack of standardization, complexity, lack of clinical evidence, and the cost of implementation [23].

The application of molecular biological techniques has allowed the production of specific antigens in large quantities for use in immunologic techniques, including recombinant antigens and synthetic peptides [24]. Molecular biology methods are characterized by a high specificity and sensitivity, particularly during the acute phase, and hybridization techniques have been used for the detection of specific DNA fragments of the parasite genome by polymerase chain reaction (PCR) [25–27]. One of the major limitations of the PCR technique in the diagnosis of Chagas disease is its low sensitivity in the chronic phase due to the very low level of circulating parasites, since these are confined to tissues [28, 29]. However, it is important to note that the sensitivity of PCR is also influenced by the method of DNA extraction and the volume of blood that is used for DNA extraction [30]. Parasite detection from organ biopsies is indeed more successful during chronic infection when parasites sequester within organs; however, this technique is impractical because biopsies are not easily collected [31, 32]. Some authors proposed that circulating parasite antigens could be used as highly specific biomarkers of infection by *T. cruzi* as observed in mouse models [33]. Serum proteins have also been proposed as markers of Chagasic patients [34]. However, diagnosis remains mostly focused on the identification of antibodies against the parasite (**Table 1**) [35]. The nature of the antigens used in anti-*T. cruzi* assays is critical for the specificity of the assay, particularly in the case of individuals infected with related protozoan parasites such as *Leishmania*, as epitopes may cross-react with crude *T. cruzi* antigens [36]. Various types of serologic tests are currently used to establish the diagnosis of Chagas disease (**Table 2**), based on total parasite extracts and/or recombinant antigens [37, 38]. These serological assays for detecting antibodies to *T. cruzi* are generally classified as screening or confirmatory assays (**Table 2**). First-line screening assays provide the presumptive identification of antibody-reactive specimens, and supplemental assays are used to confirm whether samples found reactive with a particular screening assay do indeed contain antibodies specific to *T. cruzi*. When a single screening assay is used for testing in a population with a very low prevalence of Chagas disease, the probability that an individual is infected when a reactive test result is obtained (i.e., the positive predictive value) is very low, since the majority of individuals with reactive results are not infected. This problem occurs even when an assay with high specificity is used. Accuracy can be improved if a second supplemental assay is used to retest all those specimens found reactive by the first assay. Those found non-reactive by the assay are considered negative for antibodies to *T. cruzi*. Serum/plasma samples with low antibody titer are frequently found in individuals from endemic regions. In general, those samples are difficult to confirm and give a definitive final status. The clinical significance of these samples and associated potential risk of transmission by an individual presenting with low antibody titer is also little understood. These could represent individuals spontaneous cure or treated by current

Diagnostic methods in the acute phase
Direct methods without concentration
Direct methods of concentration
• Microhematocrit • Strout test
Diagnostic methods in the chronic phase
Serology • ELISA • Indirect immunofluorescence • Indirect hemagglutination
Other parasitological and molecular biology methods* • Xenodiagnostic • In vitro culture • PCR

*Can be used in any phase, with low parasitemia, and is not detected by other methods.

Table 1.
Diagnoses for chagas disease.

antiparasitic medication [39] or have cross-reactivity with other agents such as *Leishmania* [40]. To date, and according to the WHO, an individual is diagnosed as infected with *T. cruzi* in the chronic phase of the disease when the results of two serological tests are positive, due to the different immunogenicity of different strains of the parasite, different immune responses between patients, and the existence of cross-reactions with other trypanosomatids coexisting in endemic areas when using crude parasite antigens [16, 38]. When inconclusive or discordant results appear, a third technique [38] or additional samples are required [41]. However, during the diagnosis of the disease, we must take into account certain conditions and/or variables that are not directly dependent on the design and development of the test; as in the case of the host response, this depends partly on the strain of parasite and secondly on the genetic background of the host [42]. Moreover, the existence of the "immunological memory" in models where there was a parasitological cure suggests that parasite antigens may persist in some organs, which could be inducing the production of antibodies, regardless of the presence of live parasite [43, 44]. In patients treated during the chronic phase, the objective is to analyze a tendency for negativization of the serological tests, which requires to monitor patients for many years following treatment. This has brought controversies regarding the criterion of a serologic cure [45, 46]. Some researchers believe that a reduction in antibody titers after a prolonged time can be considered as a criterion of cure, in contrast to others who recommend total negativization of serological tests [47]. In areas where Chagas disease is endemic, the choice among ELISA, IIF, or IHA for serological testing is based on availability [31, 32, 38, 48]. However, these techniques may be unspecific as they can cross-react with other parasites [49]. Because of this cross-reaction, the use of antigen secretion/excretion of parasite in diagnostic tests has been proposed. It has been shown that proteins from trypomastigotes are better antigens to detect antibodies against the parasite; however, most antigens used for immunological tests are total protein extracts derived from epimastigotes, because of the ease of

Test	Antigen	Manufacturer	Country
Immunoenzymatic assay			
AccuDiag™ Chagas ELISA Kit	NI	Diagnostic Automation/ Cortez Diagnostics, Inc.	USA
ImmunoComb® II Chagas Ab Kit	RA	Alere Inc.	Germany
Anti-Chagas IgG ELISA Kit	NI	Abcam	United Kingdom
Abbott ESA Chagas	Purified antigens	Abbott Laboratories	USA
Abbott PRISM Chagas	Purified antigens	Abbott Laboratories	USA
T. cruzi Ab (Chagas)		Bioars	Argentina
EIAgen *Trypanosoma cruzi* Ab	Total extract	Adaltis	Italy
Chagas screen ELISA	Cytoplasmic membrane antigen	Wiener lab	Argentina
Chagas ELISA IgG + IgM	RA	Vircell	Spain
Pathozyme Chagas	RA	Omega Diagnostics Limited	Scotland
Chagas Rec ELISA	RA	Human Diagnostics Worldwide	Germany
Chagas ELISA	Total extract	Ebram Produtos Laboratoriais Ltda	Brazil
Chagatek ELISA	Purified antigens	Laboratório Lemos SRL	Argentina
NovaLisa Chagas (*Trypanosoma cruzi*)	NI	NovaTec Immundiagnostica	Germany
Premier Chagas IgG ELISA test	Purified antigens	Meridian Diagnostics	USA
Test ELISA para Chagas	Total extract strain (Tulahuén and Mn)	BIOSChile	Chile
Bio-Manguinhos EIA	Total extract/RP	Bio-Manguinhos	Brazil
IVD ELISA	NI	IVD Research Inc.	USA
DRG®*Trypanosoma cruzi* IgG	NI	DRG International Inc.	USA
Chagas IgG ELISA	RA	Gull Laboratories Inc./ Meridian Bioscience Inc.	USA
Cellabs *T. cruzi* IgG CELISA	NI	Cellabs Pty Ltd.	Australia
BIOELISACRUZI	Total extract	Biolab-Mérieux	Brazil
Dia Kit Bio-Chagas	RA	Gador SA	Argentina
BIOZIMA Chagas kit	Purified antigens	Laboratório Lemos SRL	Argentina
Abbott Chagas Anticorpos EIA	RA	Abbott Laboratories	USA
Cruzi TEST ELISA	NI	GenCell Biosystems	Ireland
Chagas test *IICS*, ELISA	Total extract (Y strain)	IICS Univ de Asunción	Paraguay
HBK 401 Hemobio Chagas	Total extract	Embrabio	Brazil
Chagatest ELISA	Total extract/RP	Wiener lab	Argentina
Bioelisa Chagas	Synthetic peptides	Biokit	Spain
Chagas Hemagen	Purified antigens	Hemagen Diagnósticos	USA

Test	Antigen	Manufacturer	Country
BioMérieux	Total extract/RP	BioMérieux	France
BLK	Total extract	BLK diagnostics	Spain
Siemens IMMULITE Chagas IgG	RA	Siemens Healthcare	USA
Anti-Chagas Symbiosis	NI	Symbiosys	Brazil
ORTHO *T. cruzi* ELISA Test System	Total extract	Jhonson and Jhonson	USA
Certest	(Strain Tulahuen and Mn)	Abbott Laboratories	Spain
ImmunoComb II Chagas Ab	RA/peptides	Orgenics	Israel
Elecsys Chagas assay	Recombinant antigens	Roche Diagnostic	
Gold ELISA Chagas	Recombinant proteins and purified lysates		Brazil
ELISA Chagas III	Total extract	Grupo Bios	Chile
Imuno-ELISA Chagas	Recombinant antigens	Wama Diagnóstica	Brazil
T. cruzi Ab, DIAPRO	Recombinant antigens	Diagnostic BioProbes	Italy
Trypanosoma cruzi IgG ELISA Kit	Total extract	MyBiosource	USA
Chagas (*Trypanosoma cruzi*) IgG assay	NI	DEMEDITEC Diagnostics GmbH	Germany
Chagas (*Trypanosoma cruzi*) IgG ELISA	NI	GenWay Biotech, Inc.	USA
Chagas (*Trypanosoma cruzi*) IgG ELISA	NI	IBL International GmbH	Germany
CELQUEST CHAGAS ELISA	Recombinant antigens	ATGen Diagnostica	Italy
Immunochromatographic assay			
OnSite Chagas Ab Rapid test	RA	CTK Biotech	USA
Chagas AB Rapid	RA	Standard Diagnostics	Korea
WL Check Chagas	RA	Wiener Lab	Argentina
Chagas Instantest	Antigens attached to colloidal gold	Silanes	Mexico
Prueba rápida Chagas	Antigens attached to colloidal gold	Amunet Labarotarios	Mexico
Chagas Detect™ Plus	Multi-epitope recombinant antigen	InBios, Inc.	USA
Chagas-certum	Antigens attached to colloidal gold	Certum® Diagnostics	Mexico
Chagas Quick Test	Multi-epitope recombinant antigen	Cypress Diagnostic	Belgium
Chagas Stat-Pak assay	RA	Chembio Diagnostic Systems	USA
PATH-Lemos rapid test	RA	Laboratório Lemos SRL	Argentina
Immu-Sure Chagas *(T. cruzi*)		Millennium Biotech	USA

Test	Antigen	Manufacturer	Country
SD Chagas Ab Rapid	RA	Standard Diagnostic	Korea
ICT Operon	Purified antigens	Operon	Spain
Hemagglutination assays			
Chagas HAI Imunoserum	Sheep red blood cells are sensitized by binding *T. cruzi* antigen	Laboratório Lemos SRL	Argentina
ID-PaGIA, version 2 Ag/version 3 Ag	RA/purified antigens	DiaMed	Switzerland
Chagatest IHA	Erythrocytes sensitized with parasite lysate	Wiener	Argentina
Chagas HAI	Bird red blood cells are sensitized by binding purified *T. cruzi* (Y strain)	Ebram	Brazil
Imuno-HAI Chagas	Bird red blood cells are sensitized by binding purified *T. cruzi* (Y strain)	WAMA	Brazil
Chagas Hemagen HA	Human red blood cells are sensitized by binding epimastigote and amastigote forms of *T. cruzi* (Y and CL strain)	Hemagen Diagnosticos	USA
Hemacruzi	Erythrocytes sensitized with parasite lysate	Biolab-Mérieux	Brazil
ID-Chagas antibody test	Gel particles coated with peptides	DiaMed-ID	Switzerland
Serodia Chagas	Gelatin particles coated with inactivated *Trypanosoma cruzi* antigens	Fujirebio, Inc.	Japan
Immunofluorescence assays			
Chagas IFA	NI	Vircell	Spain
Inmunofluor Chagas kit	Epimastigotes	Biocientífica S.A	Argentina
Kit Trypanosomiasis IFI	Epimastigotes	Tryniti-Mardx (Inverness Medical)	USA
Inmunofluor Chagas IFI	Parasites NI	Biocientífica S.A. (Inverness Medical)	Argentina
MarDx IFA		MarDx Diagnostics, Inc.	USA
IFA Kit Trypanosomiasis		Innogenetics Ibérica	Spain

Test	Antigen	Manufacturer	Country
Chemiluminescent immunoassay			
Architect Chagas assay (prototype) immunoparticles	RA	Abbott Laboratories	Spain
CHAGAS VIRCLIA	NI	Vircell	Spain
PCR assays			
T. cruzi OligoC-TesT	NA	Coris BioConcept	Belgium
AMPLIRUN® TRYPANOSOMA DNA CONTROL	NA	Vircell	Spain
Loop-mediated isothermal amplification (LAMP) assay	NA	Eiken Chemical Company	China
RealCycler CHAG	NA	Progenie Molecular	Spain
TCRUZIDNA.CE	NA	Diagnostic Bioprobes Srl	Italy
RealStar® Chagas PCR Kit RUO	NA	altona Diagnostics GmbH	Germany
VIASURE *Trypanosoma cruzi* Real Time PCR Detection Kit	NA	Certets Biotec	Spain
Confirmatory assays			
Radioimmunoprecipitation analysis [RIPA]	Radiolabeled *T. cruzi* surface antigens	University of Iowa	USA
INNO-LIA Chagas assay	Recombinant and synthetic *T. cruzi* antigens	Innogenetics	Belgium
IF Imunocruzi	Epimastigotes	Biolab Mérieux	Brazil
TESA-blot	Excreted-secreted antigens	Biolab Mérieux	Brazil
Multiplex Immunoassay Multi-cruzi (prototype)	Protein array	NA	NA

RA, recombinant antigen; NI, not indicated; NA, not applicable.

Table 2.
List of commercial diagnostic tests for the serological detection of T. cruzi (Chagas disease).

obtaining them, at lower cost and the presence of common antigens with trypomastigotes [50]. Nevertheless, these tests also show cases of false negatives and cross-reactivity [51–56]. Due to the complexity of the interaction of *T. cruzi* with its host, a single recombinant antigen has not reached the efficacy shown by the total extracts of the parasite. Thus, the antigenic composition of the tests based on recombinant antigens includes a combination of several epitopes [57]. Flow cytometry has mainly been used for differential diagnosis between Chagas disease and leishmaniasis owing to cross-reactivity. Even with optimized serological assays that use parasite-specific recombinant antigens, inconclusive test results continue to be a problem [58]. However, this technique is utilized for monitoring treatments rather than for diagnosis [59]. In the past decade, several technologies have emerged as diagnostic tools capable of improving diagnosis by using several antigens. The diagnostic process becomes faster and less expensive, and the hands-on time in laboratories decreases substantially since these platforms can be fully automated. A multiplex assay platform was evaluated to detect *T. cruzi* infection using the recombinant antigens CRA, FRA, CRA-FRA fusion and parasite lysate; these antigens presented different

sensitivity and specificity by themselves; however, when mixed they increased its sensitivity and specificity, suggesting that they could be an alternative to single-test detection for Chagas disease [60]. Moreover, the immunochromatography represents a promising method which can be performed with whole blood, and it has many advantages over most existing diagnostic methods, requires little time, and does not require trained personnel; its main advantage is that it can be used in field work [61], but there are cases of discordance [62]. However, despite that, there are reports where it manifests that it no longer needed investing so much in research and development for the diagnosis of Chagas disease, because that rapid tests on the market are sufficiently valid both in America and in Europe and Asia-Pacific [63]; however, this is not entirely true, since several studies have shown discordance between the various commercial techniques. As mentioned, the diagnosis of Chagas disease is based mainly on serological tests because parasitemia is generally low or cannot be detected during the chronic phase of the infection. Finally, low reaction samples may not be detectable by all serological assays; besides, the presence of the so-called "serosilent" infections [64], in which parasitemia is detectable in seronegative individuals, represents a potential risk to acquire the parasite.

2.2 The world of diagnosis for Chagas disease: a discordant paradise

The accurate diagnosis of *T. cruzi* infection is pivotal to the clinical management of Chagas disease. *T. cruzi* has a complex life cycle, and its ability to infect any nucleated cell complicates diagnosis [65]. Additionally, the absence of a "gold standard" test that reliably and consistently detects the presence of a *T. cruzi* infection makes the evaluation of current methods difficult [28, 66]. Serodiscordance in Chagas disease remains a challenge since individuals with inconclusive results are clinically complicated to manage [67]; this problem usually arises in the diagnosis during the chronic phase. Performing two or more serological testing does not guarantee that the result shall be univocal. It is called serodiscordance when in the same patient two tests give different results (frequent situation during treatment, in pregnant patients or patient in acute cases), because the main criterion for "cure" has been the conversion to negative serology on all tests performed. However, this result is often not observed until 8–10 years posttreatment and then only in approximately 15% of treated adult subjects [68]. Experts recommend that the IFI which has a sensitivity of 95% and a specificity of 100% is used. However, it is often not the most advisable since reading can be subjective, depending on the experience of the technician. Another situation that often occurs in reading spectrophotometric tests, such as ELISA, is that the values of optical density (OD) are very close to the cutoff value (±10%), in which case the result should not be considered positive or negative but rather indeterminate. Alternatives have been proposed as IFI, antigens' parasite excretion-secretion (TESA) used in ELISA or immunoblot assays or by radioimmunoassay (RIPA), or various recombinant antigens used in immunoblot [14]. To date no test alone can establish the diagnosis or confirmation of infection by *T. cruzi* nor rule out the problem of cross-reactivity; the combination of tests usually generates discrepant results, often in a limited number of cases. Some commercial tests have very limited ability to detect *T. cruzi* infection in populations of particular study; internal tests based on crude parasite antigens showed a higher sensitivity but were still unable to detect all cases of *T. cruzi* infection [69], for example, the University of Sao Paulo conducted tests to detect *T. cruzi* antibodies using the three methods IH, IIF, and ELISA; 4000 serum samples were analyzed, of which only 1901 (48%) were positive for all three tests, 718 (18%) were negative, and 1381 (35%) had a questionable or inconclusive results. The discrepancies were attributable to the type of parasite antigen; IIF detects a specific antibody that reacts with a parasite

membrane antigen, whereas HI detects an antibody that reacts with a subcellular antigen. Each of these serological reactions operates in different specificity systems [70]. Another study in Spain was found to have a higher sensitivity (97–100%), and for serological screening of *T. cruzi* infection, a combination of tests is needed [71]. Finally, in a study conducted in a rural community in Veracruz, Mexico, using a combination of five ELISA tests based on different antigenic preparations (two in-house enzyme-linked immunosorbent assay based on crude extract, Chagatest ELISA recombinant v3.0, Chagatek ELISA, and NovaLisa Chagas), a very high level of discordance (32%) was also found among the ELISA tests used, with very poor agreement among them. This showed that the commercial tests had a very limited ability to detect *T. cruzi* infection, and the in-house tests based on crude parasite antigens showed a greater sensitivity but were still unable to detect all cases of *T. cruzi* infection, even when based on a local parasite strain [69].

To date, *T. cruzi* diagnostic present problems of sensitivity, making the diagnosis confusing and often requiring additional testing [40, 70, 71]. In congenital cases, it has been observed that discordance between the samples analyzed also occurs, and discordant results were confirmed by a third diagnostic test [72]. Although numerous assays are available for diagnosing Chagas disease, no single test is considered the reference standard to confirm the diagnosis of infection by the parasite [73–76]. Serology is a useful tool in the diagnosis of Chagas disease, but in certain circumstances, none of the techniques described above serves as a marker of cure or progression of infection. Some authors mention that the Western blot is suitable for confirmation of infection by *T. cruzi*, so it is strongly recommended for confirmation and discrimination of discordant cases [22, 77], because this technique has a sensitivity of 86.6% [20, 78], 99% [79], and 100% [80, 81] and a specificity of 100%, making it more effective than techniques such as ELISA, HAI, and IIF. The adequate choice of *T. cruzi* strains as antigen source for the diagnosis of Chagas disease is still controversial due to differences in terms of accuracy reported between different diagnostic tests. The results of this study showed that the sensitivity index did not vary, with percentages of 100% for all strains in both tests. However, the specificity index for the ELISA tests showed differences between 92 and 98% but was reduced to 78–89% when the *Leishmania*-positive sera were included [82].

2.3 Looking for the correct diagnosis

After more than a century of the discovery of Chagas disease, there is no consensus on the choice of a reference technique. Studies in South America reported a high efficiency of commercial kits manufactured in this region [40, 83]. However, work carried out in Central America shows that the use of antigens prepared from *T. cruzi* strains that are isolated in these areas increases the sensitivity of antibody detection assays [69, 84, 85]. This could be due to the predominance of the lineage TcI in the region [86] and the wide expansion of genotypes TcII–TcVI reported in the Southern Cone countries [87]; but lately, the presence of additional DTUs has been demonstrated in countries such as Mexico and the United States [88, 89]. The development of new diagnostics is partially limited by the availability of well-characterized antigens, in addition to the great variability among strains and DTUs in terms of virulence, infectivity, tissue tropism, progression of disease, drug susceptibility, and geographical distribution [31, 90–92]. However, there is as yet no clear association between genetic variants of the parasite and these life history or epidemiological characteristics. Several recombinant antigens that have serologic utility have been identified. However, the most effective antigens have been those with immunodominant, repeating B-cell epitopes [93]. The use of recombinant antigens and/or synthetic peptides has been proposed [94] to improve

specificity and sensitivity, which is essential if false-positive or false-negative results are to be avoided. Peptide analysis is a technique widely used for mapping of linear epitopes in proteins from pathogens [95, 96].

The recent availability of peptide microarrays allows rapid and inexpensive serological diagnosis with high performance [97, 98]. The availability of complete pathogen genomes has renewed interest in the development of diagnostics for infectious diseases. Synthetic peptide microarrays provide a rapid, high-throughput platform for immunological testing of potential B-cell epitopes. Therefore, computational approaches for prediction and/or prioritization of diagnostically relevant peptides are required [99]. Currently, high-density peptide chips for the discovery of linear B-cell epitopes' specific pathogens from clinical samples provide the basis for the detection of biomarkers and proteome-scale studies of the immune response against pathogens [100].

The challenge for this process is to identify, by bioinformatics within a given proteome, peptides that could be good targets for a B-cell response. A number of algorithms have been developed for computational prediction of B-cell epitopes [101]. Computational prediction of B-cell epitopes is still an active research field, and a number of state-of-the-art predictors show improved performance [102–105]. As a consequence, predicting diagnostic epitopes in the context of a particular disease or infection is a complex problem, where many additional limitations are not taken into account, such as the mechanism of invasion of infectious agents, the expression pattern of parasite proteins. All these additional variables affect the outcome of the immune response and may explain the observed variability in responses [99]. In recent years, microarray technology has been of great interest, offering valuable opportunities to study the function of genes and the development of diagnostics. The main advantage of a microarray assay is to determine different analytes simultaneously, and it is more sensitive and faster than the conventional ELISA system. Peptide microarrays combined with a bioinformatics peptide selection strategy constitute a powerful and cost-effective platform for serodiagnostic biomarker screening of infectious diseases just as Chagas disease [100]. The development of efficient methods for the detection of microspots with high sensitivity and specificity will enable new applications in future studies applied to protein microarrays [106, 107].

New-generation tests with potentially improved accuracy have been developed recently. The use of a large mixture of recombinant antigens and the incorporation of different detection systems, such as chemiluminescence, increase the sensitivity and specificity of the techniques. Other advantages of new-generation tests are automation, rapidity, and high performance, such as the Architect Chagas or BioFlash Chagas (Biokit, Lliçà d'Amunt, Spain), which have improved the diagnosis of Chagas disease with innovative new tools (large mixture of recombinant antigens and chemiluminescence as detection system). Previous studies have also proposed a chemiluminescent ELISA (CL-ELISA) with purified trypomastigote glycoproteins for the detection of lytic protective antibodies against *T. cruzi* in human serum [35, 108–110]. Detection systems, such as chemiluminescence, increase light amplification and signal duration in comparison with traditional ELISAs, which may be a point in favor of these new methodologies, leading to higher accuracy in the diagnosis of Chagas disease. Further studies with other new-generation techniques with similar characteristics (recombinant antigens and chemiluminescence) are necessary [35]. The use of a single technique would reduce diagnosis costs and therefore allow the application of screening and control programs in countries where such systems have not yet been implemented. Previous studies on the cost-effectiveness of Chagas disease management have been undertaken, but the costs of different diagnostic methods have not been compared [111–114]. Thus, several groups have implemented the usage of PCR to the identification of the genetic material from the

parasite, in blood and serum samples as well as tissue samples. Several types of the PCR techniques are available to detect *T. cruzi* DNA in serum and blood samples; among them we find conventional PCR, hot-start PCR, and nested PCR. Several tools that use probes to verify the presence/absence of specific DNA are also used; such as: Southern Blot or PCR and hybridization and real-time PCR. An important advantage that the use of PCR offers as a diagnosis tool is that it allows the characterization of the circulating strains in an endemic area for Chagas disease [115]. Finally, there is a need to develop new non-serological non-PCR-based assays to address the limitations of the current methods available for *T. cruzi* detection. For this purpose, assays that detect biomarkers of Chagas disease need to be developed. Biomarker discovery studies reported for Chagas disease lead to the identification of characteristics of host origin, such as host proteins or immune markers, which were elevated in Chagas disease [116, 117]. However, one cannot exclude the possibility that these host markers could also be modulated in conditions unrelated to *T. cruzi* infection, and thus these biomarkers have limited specificity [118]. In order to overcome the issues of specificity, the detection of pathogen-specific factors would be ideal biomarkers of *T. cruzi* infection [33].

3. Conclusions

Chagas disease causes 12,000 deaths each year, and it is estimated that between 7 and 8 million people suffer. This is one of the major public health problems in Latin America. In recent decades, cases have also been detected in North America, Europe, and Asia-Pacific, mainly as a result of migration. An ideal serological test should be easy to perform in a single step, be fast, and be cheap, require no special equipment or refrigeration of reagents, and have 100% sensitivity and specificity; unfortunately, no such test exists for Chagas disease. The lack of a reference standard serological assay for the diagnosis of *T. cruzi* infection has prompted the development of new tests, which require further evaluation, so that the development of diagnostic methods for detecting *T. cruzi* infections, after more than a hundred years of its discovery, remains a challenge which depends mainly on the availability of specific high-affinity antigens. The diagnosis of Chagas disease has limitations, mainly due to the great complexity of the factors that involve it, as well as to the low sensitivity of the parasitological techniques and the low specificity of the immunological tests. Finally, the application of immunomics, which combines serology with proteomics, would help to discover genes and molecules related to the susceptibility and immunity of *T. cruzi* infection, allowing the creation of an adequate diagnosis for the disease, elucidating new therapeutic targets, and, why not, allowing the creation of a vaccine against Chagas disease.

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Conflict of interest

The authors declare no conflict of interest.

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