

# Chagas disease: an overview of diagnosis

## Abstract

Chagas disease, caused by the flagellated protozoan *Trypanosoma cruzi*, afflicts millions of people, mainly affecting poor and neglected populations. The different transmission routes, the high genetic variability of the parasite, the different detection methods as well as the distinct phases of the disease, influence negatively the diagnosis accuracy of the disease. Diagnostic tests can range from simple parasitological techniques to complex molecular and serological techniques that can be used for early diagnosis in the acute phase of the disease, the determination of congenital transmission, to determine the epidemiological behavior of the disease, to detect the presence of the parasite, both in blood transfusions, as in organ transplantation, among others. This review addresses some of the most widely used tools to detect *T. cruzi* infection in different scenarios.

**Keywords:** chagas disease, *Trypanosoma cruzi*, diagnosis

Volume 6 Issue 3 - 2018

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**Received:** May 08, 2018 | **Published:** June 07, 2018

**Abbreviations:** IF, indirect immuno fluorescence; ELISA, enzyme-linked immuno-sorbent assay; IH, indirect hemagglutination; SAPA/TS, shed acute-phase antigens/trans-sialidase; TESA: trypomastigote excreted and secreted antigen; IA: immunoagglutination assay; Ab, antibodies; Ag, antigens; PCR, polymerase chain reaction

## Introduction

Chagas disease is caused by the flagellated protozoan *Trypanosoma cruzi*, which affects several species of mammals and is considered an important zoonosis.<sup>1</sup> It's distributed throughout the American continent, from the south of the United States to the south of Argentina; most of the cases are found in poor and rural areas of Central and South America. The endemic areas closely related to the presence of the vector, which correspond to bugs of the genus *Triatoma*, *Rhodnius* and *Panstrongylus*.<sup>2,3</sup> The human is the main reservoir in the domiciliary cycle, followed by domestic animals such as the dog, the cat and some domestic rodents. Numerous species of mammals can be naturally infected in endemic areas. Therefore, animals that invade homes and peri-domiciliary areas such as raccoons and rodents can be a risk of transmitting the disease to humans.<sup>4,5</sup> Approximately 6 to 7 million people worldwide are infected with the parasite, mainly in Latin America. In addition, and it is estimated that about 12.9% of the world population, approximately 70 million people, is at risk of contracting *T. cruzi* infection.<sup>6-8</sup> Rates of higher prevalence are found in Bolivia (6.75%), Argentina (4.13%), El Salvador (3.37%), Honduras (3.05%) and Paraguay (2.54%), while in Mexico and Brazil the prevalence is low, around 1%. However, due to their large populations, approximately one third of all people infected with *T. cruzi* live in these two countries.<sup>9,10</sup> Chagas disease is also transmitted through blood transfusions, organ transplants, through the placenta and through laboratory accidents.<sup>11-17</sup>

## Epidemiology

At the end of the 1980s, the documented number of Latin American immigrants in the United States from endemic countries for

*T. cruzi* was 2.24 million, of which 1.55 million came from Mexico. More than 7 million people from the endemic countries of *T. cruzi* became legal residents between 1981 and 2005. The infection is most frequently associated with immigrants from Mexico, Central America and South America; it's estimated that there are between 100,000 and 600,000 Latin American immigrants infected with Chagas disease in the United States.<sup>18</sup> Approximately 250,000; 8,000; 200,000 immigrants from Latin America lived in Europe, Australia and Japan respectively in the late 80's.<sup>13</sup> Immigrants in Japan are mainly Brazilians of Japanese descent whose living conditions in Brazil make it unlikely that they have been infected with *T. cruzi*. In Europe, Spain has become a magnet for immigrants from Latin America.<sup>19</sup> The deaths caused by this disease are estimated between 45 thousand and 50 thousand each year, with chronic chagasic cardiomyopathy being the main cause, while mortality during the acute phase occurs in approximately 5% of children under 2 years of age, due to acute myocarditis or meningoencephalitis.<sup>20-23</sup>

## Phases of the disease

To make a good diagnosis it's necessary to consider the complexity of Chagas disease, the epidemiology, transmission and its distinct phases. Fever is often a suggestive sign of infection in the acute phase. The lesion at the site of entry of the parasite: chagoma (furunculoid lesion on the skin) or Romaña sign (entry through the conjunctiva) is present in 20 to 50% of acute cases. In children, hepatomegaly, splenomegaly, generalized subcutaneous edema or localized on the face and lower extremities have been observed in 30% to 50% of cases; and from 30 to 80% of patients develop persistent tachycardia. The manifestations of the acute phase are solved spontaneously in a period of between 3 and 8 weeks in approximately 90% of the individuals that have been infected.<sup>21,23,24</sup> The Indeterminate stage is a direct progression between the acute phase and the defined phase (symptomatic). Approximately 50 to 70% of patients in the indeterminate phase never develop lesions and remain asymptomatic. The remaining 30 to 50% of patients develop cardiac or digestive dysfunction 10 to 30 years after the acute phase.<sup>21,23,25</sup> Finally,

the symptomatic stage is characterized by palpitations, dizziness, syncope and seizures, due to acute circulatory failure caused by decreased heart rate, ventricular tachycardia or cardiac arrest. In heart failure, arrhythmia, atypical precordial pain without evidence of coronary disease and dyspnea can be observed. In the presence of megaesophagus, there is regurgitation and aspiration of food, salivary gland hypertrophy, weight loss, and cachexia, signs of esophageal rupture and increased incidence of esophageal cancer. In the case of Megacolon, there is asymmetrical distention of the abdomen, bloating, intestinal occlusion and sigmoid colon volvulus.<sup>21,23,24</sup>

## Clinical diagnosis

The clinical diagnosis of Chagas disease is mainly given by the patient's clinical history, in addition to cabinet and laboratory studies. The cabinet studies are mainly radiological (thoracic-abdominal), electrocardiogram, ultrasound and echocardiogram, which are most useful in the chronic phase of the disease. In the acute stage, studies are focused on the search for the blood parasite. Because the sensitivity of the used methods is variable, it's advisable to follow a predetermined diagnostic routine.<sup>25,26</sup> Generally the diagnosis of Chagas disease, is made by direct observation of the trypomastigotes under the microscope in a peripheral blood sample, either from a fresh sample, a smear, with the thick-film technique stained with Giemsa or after performing the Strout test.<sup>27-29</sup> Which consists of concentrating the parasites from blood obtained without anticoagulant to favor coagulation and obtain the trypomastigotes that are suspended in the supernatant: after several cycles of centrifugation, first to eliminate the residual erythrocytes and then to concentrate the parasites, finally the sediment is analyzed under a microscope. A variant of this procedure that is very useful for the diagnosis of congenital infections is the heparinized or microhematocrit capillary tube technique; this test consists of the analysis of the parasites at the interface between the formed coagulum and the plasma of several capillaries.<sup>30-32</sup> For the morphological identification and to be able to differentiate with *T. rangeli*, it is necessary to analyze the preparations stained with Giemsa: the trypomastigotes of *T. cruzi* are characterized by having a prominent kinetoplast, which gives the appearance of being above the body of the parasite. However, the correct morphological differentiation of these two species is very complicated, even by trained personnel; therefore, in these cases the use of molecular techniques is recommended for confirmation;<sup>33-35</sup> that situation mainly occurs in the acute phase of infection and in areas where both species of the parasite are co-endemic.<sup>36</sup> When parasitemia is low, which is one of the main characteristics of the chronic stage, it is essential to draw on techniques that allow amplifying some specific DNA sequence of the parasite in order to make an adequate diagnosis. The xenodiagnosis consists of using triatomines placed on the inside of the arms or legs of the patient for 30min. Afterwards, the feces of the vector are analyzed at 30,60 and 90days, in search of metacyclic trypomastigotes in movement. This technique has been modified over time, now days it can be performed artificially with the same sensitivity as a traditional xenodiagnosis, in this way; direct exposure of the patient to the triatomines is avoided. The amount of peripheral blood used is the same as that ingested by insects in the traditional way; to reach an optimal performance it is necessary that it be processed immediately or in 4 hours after it is obtained. With the optimization of artificial xenodiagnosis, it has been possible to avoid that the patient suffers from the sting of the triatomines, although the result is still obtained between 30-90days after the feeding of these insects. Some researchers have added to this technique the polymerase chain reaction (PCR) to

detect the parasite and thus increase its sensitivity and reduce the time to 30 days, namely, to earlier times.<sup>37</sup> The diagnosis of *Trypanosoma cruzi* infection is complex, mainly during the chronic or clinical phase of the disease, where the lack of apparent signs and symptoms, in addition to the low and intermittent parasitemia, leads to the need for more specific diagnostic methods than direct parasitologicals. Which are not so reliable due to its low sensitivity, so the development of serological and molecular tools have had a great boom thanks to their sensitivity and specificity.<sup>38</sup>

## Serological diagnosis

For diagnosis in chronic phase, either asymptomatic or symptomatic phases, several immunological techniques have been developed for the detection of specific IgG antibodies against epimastigotes extracts of *T. cruzi*, including the Indirect Immuno fluorescence (IF), the Enzyme-Linked Immuno-Sorbent Assay (ELISA) and Indirect Hemagglutination (IH).<sup>8,39-42</sup> The majority of immunological tests commercially available use recombinant antigens, synthetic peptides and antigens obtained from non-native strains antigenically different from local strains, which generates low specificity and sensitivity in the tests.<sup>40,43</sup> Since no single standard reference test is available yet, diagnosis should base on the presence of IgG against various *T. cruzi* antigens by using at least two serological assays with different antigens. A subject is considered infected when the results of the two serological tests are positive.<sup>44</sup> However, in some serological results, discrepancies or inconclusive results may occur. To verify these results, the use of a third technique is recommended. Several serological assays and antigens have been proposed and evaluated for this use as confirmatory or supplementary test of *T. cruzi* infection. Nevertheless, there is no an actual consensus establishing a reference technique, and no single test is considered the gold standard for unequivocal diagnosis of infection by this parasite.<sup>45</sup> The use of a quantitative method such as immunoblotting (Western blot) can be very useful on inconclusive results, mainly in countries with endemic presence of other trypanosomatids such as *Leishmania* spp.<sup>46</sup> Despite of this, the use of western blot has some disadvantages. For example, there are no commercial tests based on this technique at this moment<sup>46</sup> and it is only able to detect linear epitopes (processed antigens on MHC-I), excluding the conformational structural epitopes. The use of serological diagnosis in Chagas disease is based on the use of extracts of *T. cruzi* epimastigotes as antigens. Although these extracts show limited specificity, they have also been reported to show high sensitivity in the chronic phase of the disease, moreover they show low sensitivity in the acute phase as well as in congenital infection.<sup>47-50</sup> The Shed Acute-Phase Antigens/Trans-Sialidase (SAPA/TS) are used in these cases to increase sensitivity. These antigens are proteins released to the extracellular medium through the surface of the *T. cruzi* trypomastigotes, meanwhile in epimastigotes, SAPA is a transmembrane protein.<sup>47,51-56</sup>

A large set of extracts/antigen preparations have been used for the serological diagnosis during *T. cruzi* infection.<sup>57</sup> However, Umezawa et al. quantified the sensitivity (100%) and specificity (99.4%) of IgG from patients with Chagas disease in both the acute phase and the chronic phase in 1996. For this, they used the western blot technique and Trypomastigote Excreted and Secreted Antigen (TESA) obtained from cells LLC-MK2 in culture, infected with trypomastigotes of the Y strain of *T. cruzi*.<sup>45,47,50,57</sup> In laboratory conditions, TESA blot is considered positive when is reactive with antigens of 130-200 kDa or antigens of 150-160 kDa. However, some sera also react with bands

of 80-120 kDa that belong to Shed Acute-Phase Antigen (SAPA). Some chronic patients also react with SAPA bands plus a band of approximately 95 kDa.<sup>47,58</sup> It has been reported that some members of SAPA and TESA molecules are part of *T. cruzi* transialidases, a superfamily of proteins implicated in the penetration and infection of host cells as result of the transference of sialic acid molecules from the host cell-surface glycoconjugates to its own surface mucin as glycoprotein. Transialidases are highly immunogenic, and both the C-terminal and the N-terminal regions stimulate strong humoral responses (B-cells); they are predominant antigens on the surfaces of bloodstream trypomastigotes, metacyclic trypomastigotes, and intracellular amastigotes.<sup>53,59-61</sup>

Despite of the fact that over time a large number of serological methods have been developed for the detection of *T. cruzi* in the late stages of Chagas disease, there are also classical and effective methods with acceptable sensitivity and specificity. For example, the Immunoagglutination Assay (IA), which is faster and less expensive than the above-mentioned test.<sup>62,63</sup> The IA for the detection of antibodies (Ab) comprises mixing serum or plasma with a suspension containing antigens (Ag) bound to latex particles. This method uses small volume samples, obtaining results in short times (5min approximately), is easy to implement and it does not require sophisticated equipment. Furthermore, it's a technique that may be used almost anywhere, when is possible detected visually the immunoagglutination reaction.<sup>62</sup> However, when some total parasite extracts are used, this test also generates cross-reaction with homologous organisms such as *T. rangeli* and *Leishmania* spp.<sup>64,65</sup> At the present, one of the most accepted and used tests for the detection of *T. cruzi* infection in samples of patients suspected of having Chagas disease, as well as in epidemiological studies, is indirect immunofluorescence (IF).<sup>44</sup> The detection of antibodies (IgG) against *T. cruzi* and the parasite's presence for prolonged periods, stimulates the prevalence of this disease.<sup>66,67</sup> In addition to the use of IgG antibodies, is possible to use IgM antibodies. These types of immunoglobulins are the first to appear during an adaptive response and are of limited duration. Because of the latter, the use of specific IgM antibodies against *T. cruzi* indicates an acute phase of Chagas disease, even in urine samples (80-kilodalton *Trypanosoma cruzi* antigen).<sup>67-69</sup>

In addition to the use of immunofluorescence and IgM as an early marker of infection, its main application is in the detection of congenital infections. The pentameric nature of IgM prevents it from traversing the intact placenta.<sup>70</sup> In addition, the fetus is capable of generating IgM antibodies by itself in response to foreign agents.<sup>67</sup> Therefore, the detection of IgM antibodies against *T. cruzi* detected in the blood of newborns is an indicator of a transplacental infection.<sup>67,68</sup> Nevertheless, false positives have been observed due contamination of IgM antibodies from the mother to the fetus, coming from damage to the integrity of the placenta and through maternal blood during childbirth.<sup>67,71,72</sup> Despite the diagnostic advances and the fact that serological methods are in many cases sensitive and specific, it's necessary to perform complementary tests (xenodiagnostic, PCR, electrocardiogram, echocardiogram, etc.) for the validation of the serological results.

## Molecular diagnosis

During Chagas disease chronic phase, serologic diagnosis methods are needed to identify infected subjects. These methods involve the *T. cruzi* indirect detection by the searching of antibodies against the parasite.<sup>73</sup> Indirect detection in serologic methods represent a

disadvantage due to the potential anti- *T. cruzi* antibody transference from mother to fetus (congenital transmission), as well as a possible crossed-reaction between other tripanosomatids such as *Leishmania* spp. and *T. rangeli* which can cause false positive results.<sup>74-76</sup> Thus, several groups have implemented the usage of PCR (polymerase chain reaction) to identification of the genetic material from the parasite, in blood and serum samples as well as tissue samples.<sup>77-80</sup> Several types of the PCR techniques are available to detect *T. cruzi* DNA in serum and blood samples, among them we find: conventional PCR that amplifies a specific sequence from the parasite's DNA, for this it's common the use of repeated sequences or DNA from kinetoplast (kDNA).<sup>77,81,82</sup> Hot-Start PCR which is a modification to the conventional PCR to diminish amplification of unspecific products;<sup>40</sup> nested PCR is employed to amplify DNA sequences that are found in very low quantities in the parasite genome enhancing the sensitivity in the system, it consists in the extension of a specific sequence in two successive amplification steps.<sup>83</sup> Several tools that utilize probes to verify presence/absence of specific DNA are used too (Southern Blot or PCR and hybridization)<sup>84</sup> Finally, real-time PCR usage allows to determine the parasite load by the quantification of the specific sequence amplification.<sup>40,77,85</sup> An important advantage that the use of PCR offers as a diagnosis tool is that allows the characterization of the circulating strains in an endemic area for Chagas disease.<sup>86</sup> Due to the wide genetic diversity in *T. cruzi*, the different strains of the parasite have been classified in six Discrete Typing Units (DTU). These differences are shown in the biological, pathological and immunological behavior as well as in the eco-epidemiology of the disease.<sup>87</sup>

The utilization of this technique to diagnostic purposes remains limited since it implies a higher cost than the conventional serological tools and requires trained and specialized staff. Nonetheless, it's used in very concrete cases like mother-fetus transmission as a confirmatory test in cases where serological evidence is not concluding and for research purposes too.<sup>40,78,83,88-90</sup> Lacking of a gold standard test as well as a biological marker that allow the identification of *T. cruzi* infection points toward the pursuit of several serological (like antigens) and/or molecular markers (like specie-specific genomic sequences) that enable a more reliable and accurate diagnosis. One of the major challenges is the great genetic diversity between the strains of the parasite since this suggests the existence of a distinct antigenic repertory among the different strains which is reflected, i. e., in the presence of immunological markers related to DTU.<sup>91-95</sup>

Diverse strategies have developed in order to identify antigenic proteins in the parasite that could work as potential candidates for a diagnosis tools and vaccines. A pursued characteristic is that the markers be "universal", which means markers must be likely to be located in every strain of the parasite, no matter the genotype it belongs. One of the most employed is the usage of libraries, from complementary DNA expression libraries to peptides libraries, however here we found a weakness: the number of proteins to be identified by this method can be low.<sup>96-113</sup> Accession of massive sequencing techniques has broadened the repertory of probable candidates that can be useful for serological diagnostic of Chagas disease.

Implementation of microarray and immunoprecipitation assays coupled to mass spectrometry, besides the availability of parasite genome project, make possible to increase the number of antigenic proteins identified.<sup>114,115</sup> Nevertheless, this feature also signifies a difficulty due to a wide number of candidate proteins that are obtained;



hence, it's necessary to make use of bioinformatic tools that allow filtering the list of candidate peptides to be analyzed so in this way the potentially reactive epitopes can be enriched. A great advantage in this kind of assays is the utilization of biological samples derived from infected subjects with the parasite. Recent use of linear epitope mapping in the antigenic proteins, supported in peptide micromatrix platforms, sets the facility to use quick serological screening.<sup>116</sup> However, the difficulty that this method presents is the use of in silico tools, which although are efficient to identify the “theoretical” antigenic properties in the parasite proteins, do not assure this antigens act like that in the biological context.

## Conclusion

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. Molecular techniques, such as the polymerase chain reaction (PCR), which detect specific and repeated DNA sequences of the parasite, represent a suitable alternative for diagnosis in some situations, particularly in acute cases, in congenital transmission and in the evaluation and control of treatment. The PCR technique also has some limitations in terms of cost, necessary infrastructure and sensitivity in the chronic phase of the disease. To obtain better results in the diagnosis, it is necessary to combine the use of different parasitological, immunological and molecular tools according to the phase of the disease that the patient faces in each particular case.

## Acknowledgements

None.

## Conflict of interest

The author declares no conflict of interest.

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