Diagnosis of Chagas Disease

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Abstract Diagnosis of Chagas disease is related to the phase of this protozoan infection. For acute phase, parasitological methods are preferred and for the chronic phase, serological ones. Parasitological methods comprise from the simplest wet smear, going through alternatives as concentration methods and tests that involve the multiplication of the parasite in media (hemoculture), triatomine insects (xenodiagnosis), or animals (inoculation of susceptible mammals), to more sophisticated molecular tests as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). All have indications, advantages, and disadvantages, as well as costs, all of which will be detailed in this chapter. Among serological tests, a vast repertoire has been also developed and standardized, from the simplest indirect hemagglutination to the sophisticated CMIA, including indirect immunofluorescence, ELISA, and rapid tests. As all are indirect tests, it is recommended to use at least two of them for a concluding laboratory result, in order to confirm or exclude the infection by *Trypanosoma cruzi*. Diagnosis may be used in different contexts as confirmation of the infection, exclusion of blood donors, epidemiological survey, congenital infection, or follow-up after specific treatment. In order to have a precise diagnosis, it is necessary to have commercial kits of proved performance and good laboratory practices, for which permanent training of laboratory personnel is mandatory. An external quality control will prove that these conditions have been fulfilled.

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1 Introduction

Diagnosis of an infectious disease involves mainly three aspects: epidemiological, clinical, and laboratory tests. All of them should fit, and this applies also to Chagas disease [1]. The epidemiology is important as infected people come from endemic areas, and depending on the prevalence of the infection in each area, the probability to obtain a positive result changes. Also it is important to question the patient if he/she has relatives with the infection under suspicion. In endemic areas, up to 2/3 of them recall the mother or grandfather or siblings infected. For non-endemic areas, the fact to traveling to an endemic area or of have being born in areas of endemicity is also helpful. Some characteristic clinical findings that are frequent in *T. cruzi*-infected people are of utmost importance, such as the complete right bundle branch block at an electrocardiogram and the occurrence of megaesophagus or megacolon. In endemic areas, more than 90% of the patients with one of these manifestations are infected. Finally, the laboratory analyses will close the diagnosis, showing that parasitological and/or serological tests are positive.

The natural history of the infection is worth to describe in order to understand some characteristics related to diagnosis. The acute phase lasts for 60 days after symptoms start and is characterized by a high parasitemia, with easily detected parasites in any drop of blood. Nevertheless, symptoms are usually scarce, and more than 90% of the infections are not detected, because the physicians do not suspect them or because the symptoms may be only fever, which subsides in few weeks. Recovery of the acute phase is the rule, and a decrease in the number of parasites easily detected is observed after the first 4 weeks. Lethality is below 5%, mainly in young children or after a transfusion or by oral route, conditions in which parasitemia is exceedingly high. After the acute phase, those infected start a silent chronic phase, with few or no parasites detected in blood, a reason for asking indirect methods for diagnosis. This is the indeterminate or asymptomatic form of the chronic phase, in which only antibody detection is sensitive enough for accurate diagnosis. An estimated 2% per year goes through symptomatic forms as the cardiac or megaesophagus and/or megacolon. Nearly half of all infected individuals never will present overt disease and will be dying of other causes. In many cases, diagnosis occurs only when individuals donate blood or when they submit themselves for routine checkups [2]. The acute phase comprises infection acquired through the vector (kissing bug) which is the more frequent route in endemic countries, as well as infection acquired through blood transfusion or organ transplantation. The oral route may be another way, by consuming drinks or food in whose preparation contaminated insects or their fecal samples were included; these occur as micro-epidemics when a number of people have been contaminated by the same preparation. Another mechanism is the vertical transmission from an infected mother to her newborn, causing congenital Chagas disease; nearly 5% of chagasic women may transmit *Trypanosoma cruzi* to their offspring, and the medical importance is that all newborns diagnosed may be cured by specific treatment [3]. Another possibility is the reactivation of an infected individual submitted to immunosuppression by corticoids or antineoplastic drugs or after acquiring HIV

infection with low CD4 counts [4]. The suspicion of these epidemiological circumstances, together with clinical signs as fever, allows the physician to ask for parasitological laboratory tests, which will be detailed in the following sections. Nowadays, the more frequent situation is for chronic-phase infected individuals, when serological tests should be requested for confirmation.

In non-endemic regions, due mainly to migration of infected people of Latin-America to any part of the world, physicians may be aware of Chagas disease and be able to ask for proper laboratory tests to confirm or exclude the clinical suspicion [5].

Trypanosoma cruzi is not homogeneous, and there are at least six different lineages of this protozoan, named discrete typing units (DTUs TcI to TcVI) [6] related to different geographical areas, to the frequency in which any one of these DTUs is present in human infection, to clinical manifestations, and to the response to chemotherapeutic agents [6]. Other differences are under investigation as the frequency of congenital transmission and the capacity to be transmitted from blood to a receptor. The more frequent in humans are TcI, TcII, and TcV. TcI is distributed mainly to the North of Amazonas River, and children submitted to specific treatment with benznidazole underwent cure in short periods of time [7]. TcII is found mainly in Central Brazil, an area in which megaesophagus is more frequent than in other areas [8]. TcV is found in humans mainly in countries of the Southern Cone of South America (Chile, Argentina, Bolivia, Paraguay, Uruguay, and South of Brazil) and is associated with a higher congenital transmission [9]. Emigration to non-endemic countries (mainly Europe) has been mainly from people originated from the Southern Cone, and hence this is the type of Tc more frequently seen in Spain and other European countries. Migration to the United States is mainly from México [5], where TcI predominates.

2 Parasitological Tests

2.1 Direct Tests, Wet Smear; Concentration Methods, Strout and Microhematocrit

The easiest test is the wet smear, by a drop of blood (10 μL) delivered onto a slide and covered with a coverslip. The preparation should be pressed (with any object) to obtain a thin layer of red blood cells separated among them that allows to detect the quick movements of the parasite. It is necessary to examine on a microscope (10 × 40) at least 100 fields, because parasitemia may be low, mainly when symptoms started several weeks before. When this method is negative and the clinical suspicion persists, a concentration method may be applied [2].

The method of Strout is also easy, cheap, and sensitive but requires 2–5 mL of venous blood without anticoagulants [10]. Once the clot is formed, the liquid phase is transferred to a test tube and spun down for 5 min at 50–100 g. The supernatant is transferred to another tube and spun down at 400 g to allow parasites go to the bottom. This supernatant (serum) is discarded and the last drop mounted on a slide, with the same procedure as the wet smear, already described.

When few blood is available (neonates), the method of microhematocrit should be used, by filling up to four capillaries and, after spinning, looking on the interface between red blood cells and plasma in a microscope. The capillary tube may be broken at the interface and proceed as with the wet smear [11]. Special care should be taken to avoid accidental contamination, by use of personal protection equipment (PPE).

Stained smears are less sensitive and only appropriate with high parasitemias, as may be observed in reactivation (immunosuppression) or transfusional transmission. Nevertheless the thick smear (stained) used for malaria diagnosis may be useful in the field, when, instead of plasmodium, a flagellate is found. Health personnel working with malaria has been trained in some areas to be able to diagnose *T. cruzi* as well.

2.2 *Multiplication Methods: Hemoculture, Xenodiagnosis, and Animal Inoculation*

During the chronic phase, in some circumstances, it may be necessary to isolate the parasite, as on chemotherapeutic trials. The low parasitemia may be detected only by multiplication methods, i.e., from few parasites at the sample, offering them the proper conditions to multiply. As a consequence, all these methods require a time to allow *T. cruzi* to increase the original low numbers. All these methods are not routine and need to be performed in research institutions. They also are not commercially available. The main ones are hemoculture, xenodiagnosis, and animal inoculation.

Hemoculture is based on harvesting heparinized blood in special media as liver infusion tryptose. It is essential to include a rather large amount of blood (i.e., 20 mL) and exclude the plasma that has antibodies and complement, which may kill the parasite. Culture is performed in several tubes, each one with 1 mL of packed red blood cells and 2–3 mL of medium. Observations should be performed monthly, for 6 months. Contamination is a risk, and one of the disadvantages is to run all the procedure in sterility [12].

Xenodiagnosis was the first procedure used by the time the disease was described. The rationale is to feed triatomine bugs with blood of the patient. It is necessary to culture colonies of these bugs, a rather difficult task. After feeding, bugs are examined, one by one, at 30 and 60 days after feeding, looking at their feces. Usually 40 bugs are used per procedure. Formerly, bugs were applied into a box onto the arms and legs of the patient, but nowadays heparinized blood collected from the patient is offered to bugs through a latex membrane (artificial xenodiagnosis). The advantage is that bugs may be transported to endemic areas and do not need sterile procedures [13].

Animal inoculation with blood from the patient or from feces of bugs is another procedure, seldom used. Susceptible mice are employed, i.e., Balb C. Tail blood of inoculated mice should be examined daily for 1–2 months [14].

With all these methods, the positivity is low and variable (around 20%), being highly dependent on the operator skills and expertise. If the method is repeated, the

positivity increases (up to 60%), but for some patients with very low parasitemia, even repeated examinations will be always negative.

2.3 *Molecular Methods: Polymerase Chain Reaction and Loop-Mediated Isothermal Amplification*

2.3.1 Polymerase Chain Reaction

PCR has been used for sensitive detection of *T. cruzi* DNA in human blood, firstly as qualitative tool [15–17] and later on as quantitative method to estimate parasitic load, using real-time PCR technology [18–20]. There are a few procedures already standardized and validated that employ whole blood treated with guanidine hydrochloride as a chaotropic agent [19, 21]; most of them use nuclear satellite DNA (satDNA) or minicircle molecule (kDNA) as parasite molecular targets plus an internal amplification standard [19, 20, 22]. High concordance was observed between real-time PCR targeted to the abovementioned sequences [22]. Analytical sensitivity is more uniform among different DTUs for kDNA qPCR than for SatDNA qPCR, being the latter less sensitive for some TcI and TcIV strains, due to a lower gene dosage, but recent characterization of satellite sequences from a higher number of strains allowed improvement of primer/probe design and consequently sensitivity [22]. In regions where *T. rangeli* infections concur with Chagas disease [22–24], satDNA is recommended for *T. cruzi*-specific detection. An external quality control program for evaluation of *T. cruzi*-qPCR performance has been recently implemented [25].

2.3.2 Loop-Mediated Isothermal Amplification (LAMP)

LAMP is able to amplify large amounts of DNA within 30–60 min of incubation at 60–65 °C, employing a complex design of primer sequences and strand displacement Bst DNA polymerase. LAMP reagents are stable at room temperatures up to 37 °C, avoiding the need of a cold chain [26, 27]. No thermocycler is needed for the reaction, and product visualization can be done by the naked eye or followed in real time by turbidity or fluorescence using intercalating dyes. In-tube visualization may be achieved using manganese loaded calcein. A first LAMP procedure targeting 18s rDNA gene that has been evaluated in triatomine feces showed a sensitivity of 100 fg of DNA per test but was cross-reactive with *Leishmania* sp. DNA [28], and in human blood detection level, sensitivity was 50 parasites/mL [29]. A recent prototype kit for detection of *T. cruzi* satDNA in human blood samples was developed by the Eiken Company [30]; it contains dried reagents on the inside of the microtube caps. It detected 1×10^{-2} parasite equivalents/mL in blood samples anticoagulated with EDTA and spiked with known concentrations of culture parasites, when DNA extraction was done using commercial columns or rapid boil and spin method and did not amplify *Leishmania* sp. or *T. rangeli* DNAs. The method appears highly sensitive for congenital Chagas disease and immunosuppressed patients with Chagas reactivation [30].

3 Serological Tests

These are employed for the diagnosis of all infected individuals during the chronic phase. They may be divided in those conventional, which are routinely used in the last 40 years and are all commercially available, and the more recent nonconventional, some of them not commercially available. Rapid tests are also employed in special circumstances. All these tests are designed to find IgG antibodies, which are present in large concentrations and show high affinity. Other immunoglobulins may be present as well, mainly anti-*T. cruzi* IgM, which may be useful in those acute cases when parasites are not easily found. Antibodies of IgM class may be present also in some chronic patients, so they are useful only in some cases during the acute phase [1].

3.1 Conventional Methods

These tests include indirect hemagglutination (IHA), indirect immunofluorescence (IIF), and the immunoenzymatic tests of ELISA (enzyme-linked immunosorbent assay). A large experience with all of them has been built in all endemic countries, and results are comparable in different centers. Several studies and publications allowed developing better products. Good laboratory practices should be followed, which include personnel training. Kits should be of good quality and retested with each new lot of reagents. Internal and external quality controls should be employed [1].

The World Health Organization recommends to employ at least two of these serological tests in order to avoid false results. The titer of each reaction should be included, and the possibility of errors with high titers is minimized. Each laboratory should include a table with the negative values, those on the gray region, and the positive values, which may differ from laboratory to laboratory [31].

IHA is the simplest and cheaper method, with few steps, which avoids errors. Sensitized red blood cells and serum from the patient are in contact for 1–2 h; after this time if antibodies are present, the red blood cells make a net on the bottom of the tube or well, which is visually read. If the red blood cells sediment on the bottom as a point, the reaction is negative. Serial dilutions permit to get the titer of the reaction, i.e., if reaction still occurs when the serum is diluted 1/100, this is the titer, indicating for sure the presence of antibodies. This test has a good specificity (>98%) and a reasonable sensitivity (>96%) [1].

IIF is used for serological diagnosis in many infectious diseases. It has several steps and incubations, it needs fluorescent microscopy, and the reading may be subjective and time-consuming. The main advantage is the sensitivity (>99%), but the specificity is lower (>96%) mainly at lower titers. Many diseases may yield a positive result at titers below 1/160, mainly leishmaniasis. The serum is placed in contact with the epimastigote form of the parasite, for 30 min at 37 °C. After washing, a further incubation with an anti-*T. cruzi* antiserum (mainly goat) conjugated with a

fluorochrome is performed. The preparation is observed on the fluorescence microscope to look for fluorescent parasites, indicating a positive reaction. Serial dilutions are performed to obtain the final titer. Infected individuals show reactivity with dilutions of sera at the order of 1/2560. Again, good laboratory practices and reagents of recognized quality are necessary to obtain a confident result [1].

ELISA is rather similar to IIF and needs several incubations; it has a high sensitivity but lower specificity. The rationale includes the contact of serum with antigens of the parasite stick to the plastic material of a well of a microplate. After this incubation antigens are in contact with an antibody anti-*T. cruzi* conjugated with an enzyme. After washing the complex, "antigen-serum-enzyme" will react with a colorful substrate if the enzyme was not washed. The colored reaction is measured by a photocolorimeter giving a reading in optical density (OD). A scale of controls builds a figure which is the cutoff value. The OD of the sample divided by the OD of the cutoff gives a figure (index) which is considered positive if higher than 1.1. This test is more objective than IIF, and results are presented as OD or the index obtained.

For all these conventional tests, results obtained may be negative, positive, or borderline (gray zone), and two of them concordantly positive or negative assure the confidence of the results [31].

3.2 Nonconventional Methods

There are a number of recent tests based on different methodologies that have been employed for serological diagnosis of Chagas disease. The most employed one is chemiluminescence, which is commercially available and used in many blood banks (Chemiluminescent Microparticle Immuno Assay, CMIA) [32]. The sensitivity is circa 100%, but specificity may be lower, so it is essential to use this type of test together with a conventional one, mainly for ascertain diagnosis of a case. It may be used as a single test for exclusion purposes as blood banks.

Other nonconventional tests are RIPA (radioimmunoassay), which is no commercially available and used only in the United States, Western blot tests (TESA-blot), and lytic assays including flow cytometry (noncommercially available) [reviewed in [1]].

3.3 Rapid Tests

These are quick tests on the same basis than those available for other conditions as diagnosis of pregnancy, kalazar, HIV, and others. For Chagas disease a membrane is sensitized with several recombinant antigens, and a drop of serum or blood is placed in contact. After few minutes, a reaction may be seen as a band, if the test is positive. These rapid tests have a number of advantages: they may be used by any individual, may be transported to the field, and do not need special temperature conditions, and

the result may be stored together with the file of the patient. Several research works have been published [33, 34] showing reasonable specificity and sensitivity of some of them. Again, they have precise indications and should not label an individual as infected unless a second, conventional test is used in parallel.

4 Other Tests

A number of tests not based in antibodies have been described, such as skin tests (delayed hypersensitivity) and the detection of circulating antigens in serum and in urine, but for different reasons they are not employed as routine tests. The search for anti-*T. cruzi* antibodies of the IgM class may be performed by IIF, when an acute case is suspected and parasites are not found. This is an "in-house" test, not commercially available that has some pitfalls as false positives when rheumatoid factor is present. Another difficulty is to have proper controls, such as sera from acute phase patients [1].

A nanoparticle assay [Chunap] has been developed for diagnosis of congenital Chagas disease in a single urine specimen at 1 month of life with more than 90% sensitivity and more than 95% specificity. The study demonstrated that poly[NIPAm] particles coupled with trypan blue dye capture and concentrate *T. cruzi* antigens in urine, and under experimental conditions these particles protect *T. cruzi* antigens in urine from enzymatic degradation [35].

5 Different Contexts for Diagnosis and Handling

5.1 Diagnosis of Acute Phase

Acute phase is defined by the presence of easily detected parasites. This includes concentration methods, already explained, but excludes multiplication techniques, as hemocultures, xenodiagnosis, and animal inoculation, because these may be also positive in some chronic-phase infected individuals.

After an incubation period, often of some days, symptoms may appear, and by this time, parasites are present in the circulation, where may be picked up for examination. Nevertheless, a number of cases may have only slight fever and not diagnosed. The physicians in endemic areas may suspect if another case was diagnosed at the same locality some time before.

The easiest test is the wet smear, but laboratory personnel should have some training before to recognize alive parasites. If a negative result comes out, concentration techniques already described may apply. Better results are obtained when fever is present. After some weeks, parasitemia declines and will be more difficult to find patent parasitemia [1].

A special situation is the acute phase that may emerge in seronegative recipients of organs from seropositive donors. In general the Strout method is used; molecular

diagnosis may be useful to detect infection earlier; indeed PCR enabled detection of bloodstream *T. cruzi* DNA between 28 and 47 days earlier than "Strout" [36]. LAMP technology also has shown potential usefulness to follow-up these cases [30].

5.1.1 Vector Transmission

Vector transmission has been the usual mechanism of infection, with an incubation period of 7–10 days; a portal of entry may suggest the diagnosis, mainly Romaña sign, which nevertheless occurs in a minority of cases. Consequently, a large proportion of patients remain undiagnosed evolving to the chronic phase. If the parasite is difficult to find during the acute phase, these cases have frequently IgM anti-*T. cruzi*, which may help.

Direct or indirect detection of circulating parasites is the method of choice. Microscopical observation of fresh blood can reveal motile trypomastigotes. Stained blood smears allow the identification of morphological characteristics of *T. cruzi*; however these methods have only 70% sensitivity in acute infections. Concentration alternatives are employed to enhance sensitivity. Few serological methods have been developed for diagnosis of acute Chagas. An IgM-type humoral response against shed acute phase antigen (SAPA), a member of trans-sialidase family, was mostly investigated [37].

5.1.2 Oral Route and Outbreaks

Outbreaks by oral route have been recognized recently as a frequent mechanism, mainly in regions where vector transmission is under control [38–41]; a number of cases with fever, usually within the same family/school or after a social event, may indicate the presence of food-transmitted infections. As this route is very effective (is the usual way by which reservoir animals get infected), high numbers of parasites are found, and a different setup of clinical manifestations are observed, as severe digestive involvement, abdominal pain, and jaundice in some cases. Mortality is higher than by the vector route, probably due to the high numbers of parasites ingested and delay for diagnosis. Outbreaks may involve many individuals, as with sugarcane in Brazil and with guava at a school in Caracas, with more than 100 infected children [38].

Oral transmission is the most important route of infection in Brazilian Amazon and Venezuela, and reports exist from oral outbreaks in Colombia, Bolivian Amazonas, and French Guiana [38–43]. In most outbreaks molecular methods have been crucial for specific and rapid diagnosis and also for identification of the parasite genotype involved.

5.1.3 Transfusional Transmission

Transfusionally acquired infection may have a large period of incubation, whose reasons are not clearly understood and should be suspected in any case with fever after a transfusion of blood or their components. Very high parasitemias are

observed, and often the diagnosis is suspected after finding flagellates in a stained smear for differential count of leukocytes. As patients have another disease that needed a transfusion, the prognosis is poor, and they may die without recognition of the superimposed infection with *T. cruzi*. Nevertheless, it should be emphasized that only 20% of the donors transmit the parasite, probably due to the paucity of bloodstream parasites, especially in chronically infected ones [1].

5.1.4 Transmission by Organ Transplantation

Transplantation involves the transmission of organs from an infected donor and the reactivation of the infected recipient. A difference between transfusion of blood from infected donors and organ transplantation from an infected donor is that parasites are always present in organs and the chance to acquire the infection increases. Furthermore, recipients of an infected organ are usually immunosuppressed, increasing the chances of severe acute phase [44].

5.1.5 Congenital Transmission

Congenital transmission is not usual (2–10% of infected mothers), but the importance of a correct diagnosis is remarkable, since all neonates detected can be easily cured after specific treatment if diagnosis is made. The presence of anti-*T. cruzi* maternal IgGs at the first months of age raises a different approach: it is recommended to search for IgGs after the ninth month of age, when maternal IgG disappears or has such low titers that assure the lack of transmission. Conversely the presence of antibodies against *T. cruzi*, at sizeable titers, at that age is a formal indication to start specific treatment. Parasitological diagnosis may be performed at birth, but a laboratory experienced personnel is necessary and available 24 h a day, which is far from the conditions usually present in endemic areas. As mentioned before, parasitological diagnosis is operator-dependent and needs a fresh sample to enable detection of motile trypomastigote forms. Furthermore, transmission may have placed during labor, and parasites will be demonstrable only 7–10 days after, when mother and baby are far away from the hospital. In these circumstances it is more feasible to look for IgG around 9 months of age, looking for antibodies with at least two conventional serological techniques [4]. At this end, it is recommended to avoid nonconventional techniques, which may yield false-positive results (mainly CMIA), as soon as more accurate strategies become validated. As explained before, at least a conventional test should be performed together with nonconventional ones to avoid misdiagnosis that may lead to treat a noninfected baby [3, 32].

Molecular methods in newborns/neonates could allow early diagnosis and bypass loss to follow-up [30, 45–48]. Noteworthy, those newborns with clinical signs present higher parasitic loads [49]. A kit prototype based on duplex TaqMan real-time PCR (qPCR) that starts from 1 mL of peripheral blood mixed with a DNA stabilizer solution has been built and validated in binomials of Chagas disease pregnant women and their newborns residing in endemic regions [50]. The accuracy of molecular detection in cord blood is still under study [50, 51].

5.1.6 Reactivation

The presence of easily detected parasites is seen in chronic-phase infected individuals that acquire AIDS or are submitted to immunosuppression for cancer, transplantation, autoimmune diseases, or other reasons [52].

As the presence of parasites is the hallmark of acute phase, this is called a reactivation of the infection and should be handled in the same way. The difference with the other forms of transmission is that the patient is already infected, so large concentrations of anti-*T. cruzi* IgGs are detectable, as well as the parasite. A proper exclusion of *T. cruzi* infection by serology, as already explained, should be mandatory in all these cases. Low CD4 cell counts in AIDS and immunosuppression with drugs for a long time and large doses, favor increase of parasitemias, and complications (unusual in other mechanisms of transmission) may occur, like panniculitis, meningoencephalitis by *T. cruzi*, and acute myocarditis, the latter two with higher mortality.

In heart transplantation, reactivation has been earlier detected and followed up by PCR methods carried out in peripheral blood and endomyocardial biopsies [44, 53, 54]. In HIV-coinfected Chagas disease patients, molecular methods are useful for differential diagnosis of meningoencephalitis due to *T. cruzi* or toxoplasmosis allowing prompt therapy decisions [55–57].

In conclusion in all these contexts of acute infection, direct parasitological tests should be performed, which may include concentration methods. The use of serology (search of antibodies of the IgM class against the parasite) may be only complimentary in those vectorial cases in which parasites were not found. The use of IgM in congenital cases has been withdrawn because most of them lack IgM. The same applies to other modes of transmission [1]. Upon validation, molecular methods will enable to close the gap of parasitological methods sensitivity and allow early and more sensitive diagnosis.

5.2 Diagnosis of Chronic Phase

Laboratorial diagnosis in this phase is performed with indirect tests (serological) because parasites are usually absent or in such low numbers dispersed on the blood that a chance to get some of them by venipuncture in one arm is remote. Nucleic acid amplification-based techniques are more sensitive than parasitological ones but due to the low and intermittent burden of bloodstream parasites are not accurate enough for diagnosis. On the other side, serological tests may have some pitfalls, and the use of two tests in parallel (on the same collection of blood), as WHO recommends, avoids most of the problems [31]. In the evaluation of serological results, two variables should be considered: specificity and sensitivity. There are tests with high specificity and others with high sensitivity, and the purpose of the diagnosis should be established. For diagnosis of a patient, specificity should be as higher as possible, to avoid mislabeling. When the exclusion of an infected sample is the final goal, a very sensitive test should be employed, even if some will be erroneously

labeled as infected. The security of the blood is more important. What is not possible is to have nowadays a single test with 100% sensitivity and 100% specificity [1].

Cross-reactions, mainly with leishmaniasis, may be seen. The concentration of antibodies present (the titer or optical density) usually helps, because infected individuals usually have high titers and indexes. As emphasized before, the epidemiology and clinical context are very important for the interpretation of the laboratory result obtained. This is particularly important in cases of visceral leishmaniasis (kalazar) where patients have severe compromise of several organs and fevers. Because in kalazar there is a strong B-cell response (polyclonal activation), with a sizeable increase in gamma globulin, antibodies of different specificities favor cross-reactions with many infections, among them, Chagas disease. A chronic case of *T. cruzi* infection will not have fever, nor hepatic or spleen enlargement, or blood alterations, often seen in kalazar. Conventional serology usually gives false-positive results in kalazar cases, and the clinician should have this in mind [1].

Serological diagnosis may be applied with different aims and contexts. This implies a selection of tests and procedures. Some situations will be briefly described, as follows.

5.2.1 Clinical Diagnosis in a Patient

This is the common situation: the physician suspects of Chagas disease in a patient and needs to confirm the suspicion, by laboratory confirmation. According to WHO recommendation, diagnosis should be based on the positivity of at least two of the tests mentioned above [30]. Tests used should be of good specificity and ideally with high titers. To diagnose a patient as infected based on a single recombinant test may lead to a false-positive result with even legal consequences.

Demonstration of the parasite in the blood may be performed by xenodiagnosis, with the classic method (four boxes with ten parasites in each) or the artificial method; the latter has several advantages. Hemoculture shows a positivity of no more than 50% of cases. Positivity of these techniques may be increased when the examination is performed two or more times.

Molecular methods lack sensitivity at the chronic phase; different PCR strategies were evaluated, and a clinical sensitivity of around 70% was achieved when only one peripheral blood sample was analyzed. Serial sampling allowed increment of PCR clinical sensitivity [58]. These approaches could be useful in those cases with dubious results on serology if they give detectable results.

5.2.2 Exclusion on a Blood Bank

The final purpose of a blood bank is to offer a safe product, without infectious material. In this case, a test with 100% sensitivity is desirable. Such a test may be ELISA or even nonconventional commercially available, as CMIA. Provided that an external quality control exists, it is possible to use a single test, because the purpose is to exclude any donor whose serum gives signals in the system, even at a low titer or in the gray zone [1].

5.2.3 Epidemiological Survey

The purpose in surveys is to know if the infection is present in a depicted area. For operational reasons, hundreds of samples may be obtained in a single day. The rationale is to use filter paper or rapid tests, avoiding the time consumed between blood extraction, separation of sera, and labeling. Tests to be used should have high sensitivity. Those few that gave a positive result may be sorted out in a second visit which will involve only a low number of collections of blood [59].

5.2.4 Vertical Transmission

The first step is to confirm the positive serology of the mother, as for clinical diagnosis. Once confirmed, the offspring may be tested with parasitological tests at birth as already described. Wet smear, microhematocrit, or PCR may detect the parasite in the blood of the newborn, who should be immediately submitted to specific treatment. Nevertheless, for reasons already explained, this approach may not be available. If this is the case, all newborns from confirmed infected mothers should be investigated later. A good possibility is to perform PCR any time after delivery. If this is not feasible, all of them should have a collection of blood after their 8 month of life, looking for IgGs, as for clinical diagnosis.

5.2.5 Follow-Up After Specific Treatment

This is a different situation. Infected individuals that were submitted to trypanocidal drugs (benznidazole or nifurtimox; see corresponding chapter) need to be followed up for a period of time, in order to know if antibodies disappear or titers are coming down. This is attained in months for neonates or patients treated during the acute phase but demands some years for those children treated during the recent chronic phase. For those in late chronic phase, a switch may be observed after some decades. Cure is obtained when no more antibodies are present, and failure is an outcome better investigated through parasitological and/or molecular tests, when they persist or become positive after treatment completion. As complete disappearance of antibodies may take time, these cases should be investigated by as many tests as possible, of the conventional type, and three are desirable [60].

Parasitological tests have limited sensitivity, and accordingly a negative result does not necessarily mean the absence of parasites, but in contrast, a positive result indicates treatment failure. A highly sensitive and reliable method to assure cure is urgently needed. Molecular methods are useful tools for treatment monitoring [61–65]. Blood-based qPCR techniques are being consistently used to detect therapeutic response or failure in clinical trials with traditional and novel drugs, which were administered with different regimens and combinations [59, 65–69]. Most trials in chronic CD have shown a lower efficacy of ravuconazole and posaconazole in comparison to benznidazole [59, 66, 69]. However, clearance of parasitic loads exerted by drugs can be transient and lead to misleading conclusions when follow-up is performed at the short term. Ideally, molecular methods used for monitoring chronic

patients should be performed for several years after treatment to confirm or discard available data. Recent findings of dormant amastigote subpopulations, refractory to benznidazole action, may represent a key factor leading to treatment failure, which deserves further investigation [70, 71].

6 Concluding Remarks

Laboratorial diagnosis of Chagas disease is well established. For acute phase, direct parasitological tests should be employed, which are rather simple but need skilled technicians to perform them. For chronic phase, search of IgG anti-*T. cruzi* is easily performed through several conventional tests. The purpose of the diagnosis may delimitate the type of tests to be used. The use of two techniques in parallel is necessary to ascertain a positive result or exclude the infection in a patient. In order to obtain a good performance, kits of recognized quality and good laboratory practices are necessary. To fulfill these needs, internal and external quality control are imperative.

Standardized and validated, in-house and commercial PCR and LAMP methodologies have been a research priority to improve current Chagas disease diagnosis [71], especially in the following scenarios: early diagnosis of congenital infection, oral outbreaks, reactivation follow-up due to immunosuppression, and treatment response monitoring [21, 25, 29, 72]. Target product profiles (TPPs) of molecular strategies for diagnosis of *T. cruzi* infection have been addressed, pointing to the need of developing point-of-care assays [61, 73]. Their evaluation in field studies is needed to predict their usefulness in the clinical practice and for public health applications.

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